



6-1-05
Best Available Con

DAC
ZFW
E

PATENT

Docket No. E1679-00007 (formerly 111590-121 US2)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Applicant:) John Hamilton
Serial No.:) 09/851,230
Filing Date:) May 8, 2001
Group Art Unit:) 1644
Examiner:) Belyavskiy, Michail A.
For:) A Method for the Treatment and Prophylaxis of
Inflammatory Conditions

Mail Stop Petition
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

I hereby certify that this correspondence is being deposited with the United States Postal Service in an envelope bearing Express Mail Label No. EV626173985US, and addressed to Mail Stop Petition, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450 on May 30, 2006.

Signature: _____

M. Lisa Wilson, Reg. No. 34,045

Sir:

PETITION

Applicants hereby petition the Director of the U.S. Patent and Trademark Office (PTO) pursuant to 37 C.F.R. § 1.181 to determine whether the priority claim is proper in U.S. Serial No. 09/885,259, an application originally filed as a provisional application and later converted to a non-provisional application. This non-provisional application published as U.S. Patent Application Publication No. US 2002/0141994 to Devalaraja *et al.* For the sake of clarity, the non-provisional application is referred to herein as "the '994 publication." The '994 publication has been cited twice against the present application pursuant to 35 U.S.C. § 102(e). This petition is filed to determine whether the '994 publication is properly available, based solely on dates, as a reference against the present application.

06/02/2006 WABDELRI 00000007 041679 09851230
01 FC:1462 400.00 DA

Applicants believe that the propriety of the priority claim is a formal matter that may be considered by the Director on petition under 37 C.F.R. § 1.181. The Director's determination of the priority date of the '994 publication will advance prosecution at this stage of the case. In an abundance of caution, Applicants alternatively file this petition pursuant to 37 C.F.R. § 1.182 as a matter not otherwise provided for, namely to request that the Director determine the effective date of a reference cited against another application. Applicants authorize payment of the petition fee of \$400 from Deposit Account 04-1679.

Finally, this petition is being timely filed within two months of the Office Action dated March 28, 2006, since May 28 and 29, 2006 are respectively a Sunday and Memorial Day, making May 30, 2006 the due date for this petition.

Requested Relief

The question in this petition is under what circumstances can an abandoned first provisional application serve as the basis for priority for a non-provisional application converted from a later-filed, second provisional application? In the circumstances of interest to this case, the first provisional was filed more than 11 months before the second provisional and more than 15 months before the request for conversion of the second provisional application. Moreover, the first provisional application was neither itself converted to a non-provisional application nor was any other non-provisional application filed before the end of the convention year of that first provisional application that could have made a domestic priority claim. Hence, the first provisional application became abandoned after expiration of the convention year and was not subject to revival. Accordingly, the first provisional application is not available to serve as a basis of priority for a non-provisional application under these circumstances.

Because these are the circumstances under which the priority claim has been made in the '994 publication, that priority claim is improper and a determination thereof is respectfully requested.

Statement of Facts

Devalaraja filed U.S. Serial No. 60/190,842 ("Provisional A") on March 20, 2000. Exhibit 1.¹ On February 23, 2001, Devalaraja and Low filed a second provisional application, U.S. Serial No. 60/270,948 ("Provisional B"). Exhibit 2. For the sake of this petition, and not to be construed as an admission, Provisional A and B concern the same subject.

The 12-month period (*i.e.*, the convention year) following the filing of Provisional A expired on March 20, 2001. Consequently, Provisional A would have become abandoned by operation of law on March 21, 2001² under 35 U.S.C. § 111(b)(5) if no request for conversion was filed by that date. Indeed, no petition to convert Provisional A was timely filed as evidenced by the transaction history and the contents of the Image File Wrapper obtained from the PTO PAIR system. Exhibit 3.

It was not until July 6, 2001 (received by the PTO on July 9, 2001) that Devalaraja and Low timely requested conversion of Provisional B to a non-provisional application. The request included a priority claim for the non-provisional application to be accorded priority of Provisional A pursuant to 35 U.S.C. § 119(e). Exhibit 4. The conversion was granted on

¹ All exhibits were obtained from the USPTO PAIR system Image File Wrapper (IFW) and represent true and accurate copies thereof.

² As is well known, if the 12 month period ends on a weekend or holiday, the time period for requesting conversion extends to the next business day. In this case, March 20, 2001 fell on a Tuesday, so Provisional A became abandoned as of March 21, 2001.

September 5, 2001 (Exhibit 5) and Provisional B was accorded U.S. Serial No. 09/885,259 and a filing date of February 23, 2001. However, the subsequently-issued filing receipt apparently did not contain the requested priority claim as evidenced by two submissions of a "Request for Corrected Filing Receipt" (one received at the PTO on April 30, 2002 and the other on May 15, 2002). Exhibit 6. While the face of the published application contains the requested priority claim, the IFW record fails to respond to those requests. Exhibit 7.

Discussion

To simplify analysis of the priority claim in the non-provisional application, *i.e.*, the '994 publication, Applicants believe it is helpful to view the chronology of events as set forth below:

<u>Date</u>	<u>Event</u>
March 20, 2000	Provisional A filed
February 23, 2001	Provisional B filed
March 21, 2001	Provisional A abandoned
July 9, 2001	Petition to convert Prov. B to a non-provisional (with priority claim to Prov. A)

One of the most basic tenets of domestic and foreign priority systems is that the benefit of an earlier application cannot be obtained if the one-year filing deadline is missed. For provisional applications this rule is embodied in 35 U.S.C. § 111(b)(5),³ which provides that a provisional application becomes abandoned when it is not converted to a non-provisional

³The full text of 35 U.S.C. § 111(b)(5) provides:

(5) Abandonment.--Notwithstanding the absence of a claim, upon timely request and as prescribed by the Director, a provisional application may be treated as an application filed under subsection (a). Subject to section 119(e)(3) of this title, *if no such request is made, the provisional application shall be regarded as abandoned 12 months after the filing date of such application and shall not be subject to revival after such 12-month period. (Emphasis added.)*

application within one year of filing.⁴ This statute further states that once abandoned, the provisional application cannot be revived. Since Provisional A was not converted to a non-provisional application, Provisional A became abandoned on March 21, 2001 and is not subject to revival. 35 U.S.C. § 111(b)(5). Once abandoned under these circumstances, it cannot serve as the basis of priority for any later-filed application. Furthermore, as of March 20, 2001, no application had been actually filed under 35 U.S.C. § 111(a) or via the PCT upon which to predicate domestic priority rights under 35 U.S.C. § 119(e)(1).⁵ Accordingly, all domestic and foreign priority rights in Provisional A were extinguished as of March 21, 2001.

On March 21, 2001, Provisional B was not entitled to the right of domestic or foreign priority of any other application or benefit of an earlier filing date of any other domestic (U.S.) application. 35 U.S.C. § 111(b)(7). This lack of entitlement was independent of the status of Provisional A.

With respect to the conversion of Provisional B to a non-provisional application, the conversion request of July 9, 2001 was timely filed within 5 months of Provisional B's filing date (February 23, 2001). Hence, Applicants do not dispute that Provisional B was properly converted to a non-provisional application—the '994 publication.

The question is whether '994 publication may properly claim domestic priority to Provisional A, which stood abandoned for more than 3 months on the date of Provisional B's

⁴ Subject to the rule that conversion can be taken on the next available business day. Clearly not at issue here.

⁵ Applicants recognize that the provisional application need not be pending at the time such applications were filed provided that such applications were actually filed within 12 months of the provisional filing date. 37 C.F.R. § 1.78(a)(5). As discussed further herein, such applications do not include a non-provisional application that was converted from a provisional application. The requirements of 37 C.F.R. § 1.78(a)(5) address timing of the priority claim not whether a non-provisional application is entitled to make such a claim in the first instance.

conversion to the '994 publication. To be clear, this question is one of entitlement in the first instance and not simply a question of timing.⁶

First, Applicants believe that 35 U.S.C. § 111(b)(5) is clear and unambiguous on its face that 12 months after the filing date of any unconverted provisional application, that application shall be regarded as abandoned and shall not be subject to revival after such 12-month period. An application that is not subject to revival cannot have any rights therein resurrected, including the right to serve as the basis of priority for another application. Applicants have found no case law or commentary to the contrary.

Second, 35 U.S.C. § 119(e)(1) provides domestic priority for an application for patent filed under 35 U.S.C. § 111(a) if that application was filed “not later than 12 months after the filing date on which the provisional application [for which it seeks priority] was filed.”⁷ However, the '994 publication is not an application for patent *actually* filed under 35 U.S.C. § 111(a). Rather, the '994 publication was filed as an application for patent under 35 U.S.C. § 111(b)(1) and converted to a non-provisional application pursuant to 35 U.S.C. § 111(b)(5). In this regard, it is instructive (and consistent) that 35 U.S.C. § 111(b)(5) only provides that the converted provisional *may* be treated as an application filed under 35 U.S.C. § 111(a), not that it *must* be so treated. Consequently, there is no absolute requirement that a non-provisional

⁶ See footnote 5.

⁷ The relevant portion of 35 U.S.C. § 119(e)(1) provides:

(e)(1) An application for patent filed under section 111(a) or section 363 of this title for an invention disclosed in the manner provided by the first paragraph of section 112 of this title in a provisional application filed under section 111(b) of this title, by an inventor or inventors named in the provisional application, shall have the same effect, as to such invention, as though filed on the date of the provisional application filed under section 111(b) of this title, if the application for patent filed under section 111(a) or section 363 of this title is filed not later than 12 months after the date on which the provisional application was filed and if it contains or is amended to contain a specific reference to the provisional application. . . .

application converted from provisional application be treated in every respect as if actually filed under 35 U.S.C. § 111(a).

Third, the foregoing is further supported by 37 C.F.R. § 1.53(c)(3) which provides, and actually cautions, that a

“provisional application . . . converted to a nonprovisional application . . . and accorded the original filing date of the provisional application . . . will result in the term of any patent to issue from the application being measured from at least the filing date of the provisional application . . . [and] applicants should consider avoiding this adverse patent term impact by filing a nonprovisional application claiming the benefit of the provisional application under 35 U.S.C. 119(e) (rather than converting the provisional application into a nonprovisional application pursuant to this paragraph).”⁸

This rule suggests conversion is undesirable and contemplates that the earliest date available for a converted non-provisional application is the filing date of the provisional application.

Fourth, the Paris Convention provides only for twelve months of foreign priority in patent cases (Paris Convention, Article 4), whereas the foregoing scenario could effectively provide two years of domestic priority to any U.S. application. Here is how that situation arises. Suppose

⁸ The relevant portion of 37 C.F.R. § 1.53(c)(3) provides:

(3) A provisional application filed under paragraph (c) of this section may be converted to a nonprovisional application filed under paragraph (b) of this section and accorded the original filing date of the provisional application. The conversion of a provisional application to a nonprovisional application will not result in either the refund of any fee properly paid in the provisional application or the application of any such fee to the filing fee, or any other fee, for the nonprovisional application. Conversion of a provisional application to a nonprovisional application under this paragraph will result in the term of any patent to issue from the application being measured from at least the filing date of the provisional application for which conversion is requested. Thus, applicants should consider avoiding this adverse patent term impact by filing a nonprovisional application claiming the benefit of the provisional application under 35 U.S.C. 119(e) (rather than converting the provisional application into a nonprovisional application pursuant to this paragraph).

that Provisional X is filed on February 1, 2000, Provisional Y is filed on February 1, 2001 and then Provisional Y is converted to a non-provisional application a year later on February 1, 2002. All these actions would have been timely and if that were the end of the story, the non-provisional application would only have an earliest effective filing date as of February 1, 2001 with the usual one year of domestic priority. However, if the non-provisional application includes a priority claim to Provisional X, then the non-provisional application now has an earliest effective filing date of February 1, 2000 thus providing two years of domestic priority! This scenario, if allowable, would constitute a major loophole for U.S. applications and does not appear to have been intended by the Paris Convention. If such a scenario does not in fact violate the Paris Convention, it certainly violates it in spirit.

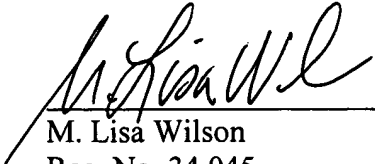
In conclusion, Applicants urge that the '994 publication is only entitled to an earliest effective filing of February 23, 2001 and is not entitled to claim priority of any abandoned provisional application that was filed more than one year prior to the date of the request for conversion. For the '994 publication, that means any provisional application filed on or before July 9, 2000 and subsequently abandoned cannot serve as the basis of a priority claim for the '994 publication. Since Provisional A was filed on March 20, 2000 and became abandoned on March 21, 2001, the '994 publication cannot claim priority thereto. Accordingly, the earliest effective filing date of the '994 publication is February 23, 2001.

Conclusion

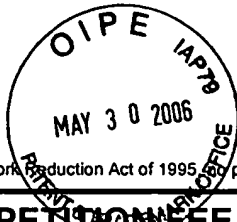
For all the foregoing reasons, Applicants respectfully request that the Director determine that the earliest effective filing date of the '994 publication is February 23, 2001.

Respectfully submitted,

Date: May 30, 2006


M. Lisa Wilson
Reg. No. 34,045

Duane Morris LLP
380 Lexington Ave.
New York, NY 10168
Tel.: (212) 692-1000
Fax: (212) 692-1020
Direct Line: (212) 691-1092
Email: lwilson@duanemorris.com



PTO/SB/17p (11-05)

Approved for use through 07/31/2007. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

PETITION FEE
Under 37 CFR 1.17(f), (g) & (h)
TRANSMITTAL
(Fees are subject to annual revision)Send completed form to: Commissioner for Patents
P.O. Box 1450, Alexandria, VA 22313-1450

Application Number	09/851,230
Filing Date	May 8, 2001
First Named Inventor	John Hamilton
Art Unit	1644
Examiner Name	Belyavskiy, Michail
Attorney Docket Number	E1679-00007

Enclosed is a petition filed under 37 CFR 1.182 that requires a processing fee (37 CFR 1.17(f), (g), or (h)). Payment of \$ 400.00 is enclosed. 179

This form should be included with the above-mentioned petition and faxed or mailed to the Office using the appropriate Mail Stop (e.g., Mail Stop Petition), if applicable. For transmittal of processing fees under 37 CFR 1.17(i), see form PTO/SB/17i.

Payment of Fees (small entity amounts are NOT available for the petition fees)☒ The Commissioner is hereby authorized to charge the following fees to Deposit Account No. 04-1679:☒ petition fee under 37 CFR 1.17(f), (g) or (h) ☒ any deficiency of fees and credit of any overpayments

Enclose a duplicative copy of this form for fee processing.

☐ Check in the amount of \$ _____ is enclosed.☐ Payment by credit card (Form PTO-2038 or equivalent enclosed). Do not provide credit card information on this form.**Petition Fees under 37 CFR 1.17(f): Fee \$400 Fee Code 1462**

For petitions filed under:

- § 1.36(a) - for revocation of a power of attorney by fewer than all applicants
- § 1.53(e) - to accord a filing date.
- § 1.57(a) - to accord a filing date.
- § 1.182 - for decision on a question not specifically provided for.
- § 1.183 - to suspend the rules.
- § 1.378(e) - for reconsideration of decision on petition refusing to accept delayed payment of maintenance fee in an expired patent.
- § 1.741(b) - to accord a filing date to an application under § 1.740 for extension of a patent term.

Petition Fees under 37 CFR 1.17(g): Fee \$200 Fee Code 1463

For petitions filed under:

- § 1.12 - for access to an assignment record.
- § 1.14 - for access to an application.
- § 1.47 - for filing by other than all the inventors or a person not the inventor.
- § 1.59 - for expungement of information.
- § 1.103(a) - to suspend action in an application.
- § 1.136(b) - for review of a request for extension of time when the provisions of section 1.136(a) are not available.
- § 1.295 - for review of refusal to publish a statutory invention registration.
- § 1.296 - to withdraw a request for publication of a statutory invention registration filed on or after the date the notice of intent to publish issued.
- § 1.377 - for review of decision refusing to accept and record payment of a maintenance fee filed prior to expiration of a patent.
- § 1.550(c) - for patent owner requests for extension of time in ex parte reexamination proceedings.
- § 1.956 - for patent owner requests for extension of time in inter partes reexamination proceedings.
- § 5.12 - for expedited handling of a foreign filing license.
- § 5.15 - for changing the scope of a license.
- § 5.25 - for retroactive license.

Petition Fees under 37 CFR 1.17(h): Fee \$130 Fee Code 1464

For petitions filed under:

- § 1.19(g) - to request documents in a form other than that provided in this part.
- § 1.84 - for accepting color drawings or photographs.
- § 1.91 - for entry of a model or exhibit.
- § 1.102(d) - to make an application special.
- § 1.138(c) - to expressly abandon an application to avoid publication.
- § 1.313 - to withdraw an application from issue.
- § 1.314 - to defer issuance of a patent.

Signature

M. Lisa Wilson

Typed or printed name

May 30, 2006

Date

34,045

Registration No., if applicable

This collection of information is required by 37 CFR 1.17. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 5 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

PROVISIONAL APPLICATION FOR PATENT COVER SHEET (Large Entity)

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53 (c).

INVENTOR(S)/APPLICANT(S)					
Given Name (first and middle [if any])		Family Name or Surname		Residence (City and either State or Foreign Country)	
Madhav Narasimha		Devalaraja		2715 Windwood Dr. #62, Ann Arbor, MI 48105	
<input type="checkbox"/> Additional inventors are being named on page 2 attached hereto					
TITLE OF THE INVENTION (280 characters max)					
COLONY STIMULATING FACTORS AS SYNERGIZERS OF CHEMOKINE INDUCED CELL RECRUITMENT AND THEIR USE TO SCREEN FOR ANTAGONISTS OF CSF RECEPTOR ACTIVITY					
CORRESPONDENCE ADDRESS					
Direct all correspondence to:					
<input type="checkbox"/> Customer Number				Place Customer Number Bar Code Label here	
OR					
<input checked="" type="checkbox"/> Firm or Individual Name		Michael J. Atkins			
Address		Warner-Lambert Company			
Address		2800 Plymouth Road			
City		Ann Arbor		State	Michigan
Country		USA		ZIP	48105
		Telephone	734-622-6218	Fax	734-622-1553
ENCLOSED APPLICATION PARTS (check all that apply)					
<input checked="" type="checkbox"/>	Specification	Number of Pages	21		
<input checked="" type="checkbox"/>	Drawing(s)	Number of Sheets	12		
<input checked="" type="checkbox"/>	Other (specify)		15 claims on 2 pages abstract on 1 page		
METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT (check one)					
<input type="checkbox"/> A check or money order is enclosed to cover the filing fees					
<input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge filing fees or credit any overpayment to Deposit Account Number:					
				23-0455	FILING FEE AMOUNT (\$)
					\$150.00
The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.					
<input checked="" type="checkbox"/> No.					
<input type="checkbox"/> Yes, the name of the U.S. Government agency and the Government contract number are:					

Respectfully submitted,

SIGNATURE Michael J. AtkinsDATE March 20, 2000TYPED or PRINTED NAME Michael J. AtkinsREGISTRATION NO. 35,431
(if appropriate)TELEPHONE 734-622-5218**USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT**

SEND TO: Box Provisional Application, Assistant Commissioner for Patents, Washington, DC 20231

PROVISIONAL APPLICATION FOR PATENT COVER SHEET (Large Entity)

INVENTOR(S)/APPLICANT(S)		
Given Name (first and middle (if any))	Family Name or Surname	Residence (city and either State or Foreign Country)

Certificate of Mailing by Express Mail

<p>I certify that this application and enclosed fee is being deposited on <u>March 20, 2000</u> with the U.S. Postal Service "Express Mail Post Office to Addressee" service under 37 C.F.R. 1.10 and is addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231.</p>
<p><i>Cindy Malocha</i> Signature of Person Mailing Correspondence</p>
<p>Cindy Malocha Typed or Printed Name of Person Mailing Correspondence</p>
<p>EJ676053163US "Express Mail" Mailing Label Number</p>

USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

SEND TO: Box Provisional Application, Assistant Commissioner for Patents, Washington, DC 20231

A0000326L1-01MJA

2000 2001 2002 2003 2004 2005 2006 2007 2008 2009 2010 2011 2012 2013 2014 2015 2016 2017 2018 2019 2020 2021 2022 2023 2024 2025 2026 2027 2028 2029 2030 2031 2032 2033 2034 2035 2036 2037 2038 2039 2040 2041 2042 2043 2044 2045 2046 2047 2048 2049 2050 2051 2052 2053 2054 2055 2056 2057 2058 2059 2060 2061 2062 2063 2064 2065 2066 2067 2068 2069 2070 2071 2072 2073 2074 2075 2076 2077 2078 2079 2080 2081 2082 2083 2084 2085 2086 2087 2088 2089 2090 2091 2092 2093 2094 2095 2096 2097 2098 2099 2100 2101 2102 2103 2104 2105 2106 2107 2108 2109 2110 2111 2112 2113 2114 2115 2116 2117 2118 2119 2120 2121 2122 2123 2124 2125 2126 2127 2128 2129 2130 2131 2132 2133 2134 2135 2136 2137 2138 2139 2140 2141 2142 2143 2144 2145 2146 2147 2148 2149 2150 2151 2152 2153 2154 2155 2156 2157 2158 2159 2160 2161 2162 2163 2164 2165 2166 2167 2168 2169 2170 2171 2172 2173 2174 2175 2176 2177 2178 2179 2180 2181 2182 2183 2184 2185 2186 2187 2188 2189 2190 2191 2192 2193 2194 2195 2196 2197 2198 2199 2200 2201 2202 2203 2204 2205 2206 2207 2208 2209 2210 2211 2212 2213 2214 2215 2216 2217 2218 2219 2220 2221 2222 2223 2224 2225 2226 2227 2228 2229 2230 2231 2232 2233 2234 2235 2236 2237 2238 2239 2240 2241 2242 2243 2244 2245 2246 2247 2248 2249 2250 2251 2252 2253 2254 2255 2256 2257 2258 2259 2260 2261 2262 2263 2264 2265 2266 2267 2268 2269 2270 2271 2272 2273 2274 2275 2276 2277 2278 2279 2280 2281 2282 2283 2284 2285 2286 2287 2288 2289 2290 2291 2292 2293 2294 2295 2296 2297 2298 2299 2300 2301 2302 2303 2304 2305 2306 2307 2308 2309 2310 2311 2312 2313 2314 2315 2316 2317 2318 2319 2320 2321 2322 2323 2324 2325 2326 2327 2328 2329 2330 2331 2332 2333 2334 2335 2336 2337 2338 2339 2340 2341 2342 2343 2344 2345 2346 2347 2348 2349 2350 2351 2352 2353 2354 2355 2356 2357 2358 2359 2360 2361 2362 2363 2364 2365 2366 2367 2368 2369 2370 2371 2372 2373 2374 2375 2376 2377 2378 2379 2380 2381 2382 2383 2384 2385 2386 2387 2388 2389 2390 2391 2392 2393 2394 2395 2396 2397 2398 2399 2400 2401 2402 2403 2404 2405 2406 2407 2408 2409 2410 2411 2412 2413 2414 2415 2416 2417 2418 2419 2420 2421 2422 2423 2424 2425 2426 2427 2428 2429 2430 2431 2432 2433 2434 2435 2436 2437 2438 2439 2440 2441 2442 2443 2444 2445 2446 2447 2448 2449 2450 2451 2452 2453 2454 2455 2456 2457 2458 2459 2460 2461 2462 2463 2464 2465 2466 2467 2468 2469 2470 2471 2472 2473 2474 2475 2476 2477 2478 2479 2480 2481 2482 2483 2484 2485 2486 2487 2488 2489 2490 2491 2492 2493 2494 2495 2496 2497 2498 2499 2500 2501 2502 2503 2504 2505 2506 2507 2508 2509 2510 2511 2512 2513 2514 2515 2516 2517 2518 2519 2520 2521 2522 2523 2524 2525 2526 2527 2528 2529 2530 2531 2532 2533 2534 2535 2536 2537 2538 2539 2540 2541 2542 2543 2544 2545 2546 2547 2548 2549 2550 2551 2552 2553 2554 2555 2556 2557 2558 2559 2560 2561 2562 2563 2564 2565 2566 2567 2568 2569 2570 2571 2572 2573 2574 2575 2576 2577 2578 2579 2580 2581 2582 2583 2584 2585 2586 2587 2588 2589 2590 2591 2592 2593 2594 2595 2596 2597 2598 2599 2600 2601 2602 2603 2604 2605 2606 2607 2608 2609 2610 2611 2612 2613 2614 2615 2616 2617 2618 2619 2620 2621 2622 2623 2624 2625 2626 2627 2628 2629 2630 2631 2632 2633 2634 2635 2636 2637 2638 2639 2640 2641 2642 2643 2644 2645 2646 2647 2648 2649 2650 2651 2652 2653 2654 2655 2656 2657 2658 2659 2660 2661 2662 2663 2664 2665 2666 2667 2668 2669 2670 2671 2672 2673 2674 2675 2676 2677 2678 2679 2680 2681 2682 2683 2684 2685 2686 2687 2688 2689 2690 2691 2692 2693 2694 2695 2696 2697 2698 2699 2700 2701 2702 2703 2704 2705 2706 2707 2708 2709 2710 2711 2712 2713 2714 2715 2716 2717 2718 2719 2720 2721 2722 2723 2724 2725 2726 2727 2728 2729 2730 2731 2732 2733 2734 2735 2736 2737 2738 2739 2740 2741 2742 2743 2744 2745 2746 2747 2748 2749 2750 2751 2752 2753 2754 2755 2756 2757 2758 2759 2760 2761 2762 2763 2764 2765 2766 2767 2768 2769 2770 2771 2772 2773 2774 2775 2776 2777 2778 2779 2780 2781 2782 2783 2784 2785 2786 2787 2788 2789 2790 2791 2792 2793 2794 2795 2796 2797 2798 2799 2800 2801 2802 2803 2804 2805 2806 2807 2808 2809 2810 2811 2812 2813 2814 2815 2816 2817 2818

COLONY STIMULATING FACTORS AS SYNERGIZERS OF CHEMOKINE
INDUCED CELL ECRUITMENT AND THEIR USE TO SCREEN FOR
ANTAGONISTS OF CSF RECEPTOR ACTIVITY

5

FIELD OF THE INVENTION

The present invention is directed to a haematopoietic factor called "colony stimulating factor" capable of synergizing the attracting capabilities of chemokines and of inducing the accumulation and in vitro and in vivo of key components of inflammatory responses.

10 Various types of agents that inhibit or otherwise hinder the production, release, or activity of CSF could be used therapeutically in the treatment of asthma and other inflammatory diseases, such as autoimmune disease, inflammatory conditions, such as sepsis, and various chronic inflammatory diseases such as rheumatoid arthritis and psoriasis. The present invention is, therefore, directed to antagonists of CSF and their use to treat, 15 prophylactically or otherwise, asthma and other inflammatory diseases. The present invention is also directed to assays for screening antagonists of CSF.

BACKGROUND OF THE INVENTION

20

Colony stimulating factors (CSFs) which stimulate the differentiation and/or proliferation of bone marrow cells have generated much interest because of their therapeutic potential for restoring depressed levels of hematopoietic stem cell-derived cells. CSFs in both human and murine systems have been identified and distinguished according to their 25 activities. For example, granulocyte-CSF (G-CSF) and macrophage-CSF (M-CSF) stimulate the in vitro formation of neutrophilic granulocyte and macrophage colonies, respectively while GM-CSF has broader activities and stimulate the formation of both macrophage, neutrophilic and eosinophilic granulocyte colonies. Macrophages, neutrophils, basophils, eosinophils, and related molecules are key components of 30 inflammatory responses.

Chemokines are chemotactic cytokines that are released by a wide variety of cells to attract macrophages, T cells, eosinophils, basophils and neutrophils to sites of inflammation. There are two classes of chemokines, the members of each class share an organizing primary sequence motif. The alpha chemokines, such as interleukin-8 (IL-8), neutrophil-activating protein-2 (NAP-2) and melanoma growth stimulatory activity protein (MGSA) are chemotactic primarily for neutrophils, whereas beta chemokines, such as RANTES (regulation-upon-activation, normal T expressed and secreted), MIP-1 alpha (macrophage inflammatory protein), MIP-1 beta, MCP-1 (monocyte chemotactic protein-1), MCP-2, and MCP-3 are chemotactic for monocytes, T-cells, eosinophils, and basophils.

The chemokines bind specific cell-surface receptors belonging to the family of G-protein-coupled seven-transmembrane-domain proteins which are termed "chemokine receptors." On binding their cognate ligands, chemokine receptors transduce an intracellular signal through the associated trimeric G protein, resulting in a rapid increase in intracellular calcium concentration. Chemokine receptors, such as CCR-1, CCR-2, CCR-2a, CCR-2b, CCR-3, CCR-4, CCR-5, CXCR-3, CXCR-4, have been implicated as being important mediators of inflammatory and immunoregulatory disorders and diseases, including asthma, sepsis, arthritis, and atherosclerosis. For example, the chemokine receptor CCR-3 plays a pivotal role in attracting eosinophils to sites of allergic inflammation (Mechanisms of enhanced lung injury during sepsis. *Amer J Pathol.* 1999, 154:1057-1065; Prevention of cerebral edema and infarct in cerebral reperfusion injury by an antibody to interleukin-8. *Lab Invest.*, 1997, 77:119-125; Chemokines and Atherosclerosis. *Curr Opin Lipidol* 9:397-405; Chemokines: Signal lamps for trafficking of T and B cells for development and effector function. *J Leuko Biol.* 65:6-15; Interleukin-8 receptor modulates IgE production and B-cell expansion and trafficking in allergen-induced pulmonary inflammation. *J Clin Invest.* 1999. 103:507-515; Differential expression of three T lymphocyte-activating CXC chemokines by human atheroma-associated cells. *J Clin Invest* 1999. 104:1041-1050.)

Accordingly, agents which modulate chemokine receptors would be useful in such disorders and diseases.

One chemokine in particular is the Interleukin 8 (IL-8) which is a cytokine that promotes the recruitment and activation of neutrophil leukocytes and represents one of several endogenous mediators of the acute inflammatory response. In the past it was variously termed neutrophil-activating factor, monocyte-derived neutrophil chemotactic factor, interleukin-8 (IL-8), and neutrophil-activating peptide-1. IL-8 has gained the widest acceptance and will be used herein.

The most abundant naturally occurring form of the IL-8 monomer is a 72-residue protein apparently derived by processing of a 99-residue precursor. Other proteins with related sequences, including neutrophil-activating peptide-2 1 ENS-78 and GRO alpha (with melanoma growth stimulatory activity) are IL-8 homologues which have neutrophil-activating properties.

Inflammation and autoimmune responses are initiated by leukocytes which migrate out of the microvasculature and into the extravascular space in response to chemoattractant molecules. These chemoattractants may be from the host and include cytokines, activated complement components or may be released from an invading organism (e.g., N-formylated peptides or MDP dipeptide). Once exposed to chemoattractants within the vasculature, the leukocytes become activated and capable of adhering to the endothelium providing the first step in the development of inflammation. Stimulated neutrophils adhere to the endothelium of the microvasculature in response to a gradient of chemoattractants which direct the cells into the extravascular space toward the source of the chemoattractant. (Anderson et al., J Clin Invest 74:536-551, 1984; Ley, K, et al., Blood 77:2553-2555, 1991; Paulson, J. C., Selectincarbohydrate-mediated adhesion of leukocytes, Adhesion: Its Role in Inflammatory Disease, W. H. Freeman, 1992; Lasky, L. A., The homing receptor (LECAM 1/L-selectin), Adhesion: Its role in inflammatory disease, W. H. Freeman, 1992.)

Subsequent observations (Buyon, J. P., et al., Clin Immunol Immunopathol 46:141-149, 1988; Abramson, S. B., et al., Hosp Pract 23:45-56, 1988; Clark-Lewis, I. et al.,

5 Biochemistry 90:3574-3577, 1993); have suggested that the endothelium has a critical role in the events leading to the development of the inflammatory lesion. This model of inflammation suggests that leukocytes are directed to an inflamed locus by stimulated endothelium. After stimulation with cytokines or bacterial products, the endothelium arrests leukocytes as they traverse (roll along) the microvasculature near sites of
10 inflammation. After being forced to stop in the microvasculature, the leukocytes are then activated to adhere more tightly to the endothelium and to migrate to the albuminal aspect of the vessel. The leukocyte, once it is out of the blood vessel is then capable of following a gradient of chemoattractants toward the exciting pathogen.

15 Vascular endothelium, activated by stimulants such as IL-1, IL-8, TNF, or LPS, appears to play a pivotal role in this process through the production of pro-inflammatory substances, including chemoattractants and cytokines.

The inflammatory properties of IL-8 were initially demonstrated from a purified natural product injected intradermally into rabbits to evaluate the proinflammatory properties, (Rampart, M. et al., Am J Path 135(1L):21-25, 1989). More recently neutralizing antibodies to human IL-8 were shown to have a protective effect in inflammatory lung injury in rats. This antibody blocked the glycogen-induced accumulation of neutrophils in rats and was protective against lung interdermal vascular injury induced by the disposition of IgG immune complexes. This latter model of injury has been shown to be E-selectin dependent. The protective effect of the neutralizing antibody correlated with reduced tissue accumulation of neutrophils as measured by myeloperoxidase content. Preliminary nonhuman primate studies have confirmed the activity of IL-8 on hematological parameters. IL-8 was administered by both bolus and continuous infusion to baboons. This resulted in a rapid, transient and severe granulocytopenia followed by granulocytosis that persisted as IL-8 levels remained detectable within the circulation.

Histopathological examination revealed a mild to moderate neutrophil margination in the lung, liver and spleen which was of greater severity in animals receiving the continuous infusion of IL-8.

- 5 High levels of intravascular IL-8 have been reported in systemic conditions such as septic shock (Danner, R. L., et al., Clin Res 38:352A, 1990). These authors have speculated that intervascular IL-8 may impair leukocyte adhesion and thus protect organs from PMN mediated injury. The intravenous administration of IL-8 induced an immediate and transient neutropenia that was similar in kinetic profile to that described with other
- 10 chemoattractants. This neutropenia was a result of pulmonary PMN sequestration and is consistent with the demonstration of abundant IL-8 receptor on PMNs. Following this transient neutropenia (approximately 30 minutes) cells recirculate with a normal half life. Shortly thereafter neutrophilia, a characteristic of IL-8, is observed. The neutrophilia likely reflects recruitment of mature PMNs to a marginal pool in the lung and other
- 15 organs as well as immature PMNs from the marrow.

- Endothelial cells can exert both proinflammatory and anti-inflammatory effects by virtue of the mediators they generate. Endothelial cells can be stimulated to generate IL-8, but unlike other mediators, IL-8 may be released from the endothelial cell. Alternatively,
- 20 endothelial cell produced IL-8 is an important chemoattractant and activator of neutrophils. There is evidence that systemic IL-8 can bind to endothelial cells which could produce a local activation of the endothelium resulting in the ability of this altered endothelium to attract neutrophils that have come into contact with the (activated) endothelium. One working hypothesis is that IL-8 initially functions as a
- 25 proinflammatory cytokine, whereas its continued generation and release from the endothelium ultimately causes a down regulation of neutrophils, with a curtailment in their further recruitment. Whether the cell associated IL-8 or released IL-8 provides the vital contribution to the outcome of the inflammatory response remains unresolved.
- 30 IL-8 binds with a higher affinity to CXCR1 and with considerably lower affinity to CXCR2 and respond with a calcium flux when bound by structurally related ligands with

the following order of potency: IL-8 > NAP-2. However, another structurally related chemokine MGSA, binds specifically to CXCR2 only but with a lower affinity than IL-8. These differences in the affinity correlate with the effectiveness of these compounds when competing with the radio labeled IL-8 for binding to neutrophils C5a, a structurally related chemoattractant that is similar in size and charge to IL-8 and which has a receptor in the same family, does not activate the IL-8 receptor.

The in vitro effects of IL-8 on neutrophils are similar to those of other chemotactic antagonists such as C5a and fMet-Leu-Phe and include induction of a transient rise in cytosolic free calcium, the release of granules containing degradative enzymes such as elastase, the respiratory H₂O₂ burst, neutrophil shape change, and chemotaxis. IL-8 appears to bind to two or more receptor sites on neutrophils with a frequency of approximately 64,000/cell and a K_d of 0.2 nM.

As it is established that IL-8 is a key mediator of inflammatory diseases, it would be desirable to identify substances capable of blocking or interrupting the activity of IL-8 for use in anti-inflammatory compositions. Such compositions may prove to be advantageous over presently available NSAID's, steroid based anti-inflammatory drugs and cytotoxic drugs which often have severe side-effects with the continued usage that is required for chronic inflammatory diseases. It would also be desirable to identify IL-8 analogs having an increased inflammatory activity for medical research applications.

SUMMARY

The present invention is directed to the discovery that CSFs appear to be critical for leukocyte, specifically PMN, recruitment and exhibits synergizing activity with chemokines. CSF's are defined to include the classic structurally distinguishable chemokines based on the C-terminal cysteine arrangement, as opposed to f-MLP which does not belong to the classic chemokine classes. In one preferred embodiment of the present invention, it was discovered that G-CSF appears to be critical for neutrophil

recruitment and exhibits synergizing activity with IL-8, both in vitro and in vivo. Studies indicating that neutralizing G-CSF antibodies inhibit the G-CSF induced synergism supported the G-CSF specific mechanism.

- 5 Because leukocytes are important mediators of inflammatory and immunoregulatory disorders and diseases, agents which modulate CSF or CSF receptors will be useful in such disorders and diseases. The present invention, therefore, also provides an antagonist of CSF capable of inhibiting or minimizing the attractive capabilities of chemokines for leukocytes and of inhibiting or minimizing leukocyte accumulation and/or activation in vitro and in vivo.

- 10 The present invention further provides screens or assays for identifying agents that inhibits or otherwise hinders the binding of a CSF to a CSF receptor, for example, any agent that binds to a CSF or to a CSF receptor. Further agents that have therapeutic potential are those that prevent or reduce activation of CSF receptors. Screens that can be employed in the identification of such antagonists/agonists are known to those of skill in the art. The different methods one could use to identify antagonists or agonists of CSF receptors include, but are not limited to: 1) Look for G-CSF binding to its receptor on the cells over expressing the G-CSF receptor; 2) Look for down stream kinase activations and develop a high throughput assay; 3) Look for the transcription factor activations (such as STATs) as a functional read out in reporter gene assays amenable for high throughput screening.

BRIEF DESCRIPTION OF THE DRAWINGS

- 25 Figures 1 to 11 of the accompanying drawings illustrate the present invention. The following is a brief description of the Figures. A more detailed description of the Figures is given below and in the Examples section of this specification.

- 30 Figure 1 illustrates that G-CSF synergizes IL-8 induced PMN chemotaxis.
Figure 2 illustrates that GM-CSF synergizes IL-8 induced PMN chemokines.

Figure 3 illustrates the dose response curve for IL-8 with fixed concentration of G-CSF.
Figure 4 illustrates that G-CSF does not synergize f-MLP induced neutrophil chemotaxis.
Figure 5 illustrates that G-CSF enhances IL-8 induced in vivo neutrophil intradermal recruitment.
5 Figure 6 illustrates the binding of I¹²⁵ G-CSF on polymorphonucleocytes (PMN).
Figure 7 illustrates that G-CSF neutralizing antibody inhibits G-CSF synergized chemotaxis.
Figure 8 illustrates that G-CSF pre-incubation alters PMN chemotactic response to IL-8.
Figure 9 illustrates that G-CSF does not alter IL-8 induced calcium flux.
10 Figure 10 illustrates that G-CSF does not increase IL-8 binding on PMNs.
Figure 11 illustrates that G-CSF pre-incubation does not alter IL-8 binding.
Figure 12 illustrates that G-CSF pre-incubation alters PMN response to IL-8.

15 DETAILED DESCRIPTION OF THE INVENTION

In order to provide an understanding of several of the terms used in the specification and claims, the following definitions are provided:

20 Biological activity

The term biological activity is a function or set of functions performed by a molecule in a biological context (i.e., in an organism or an in vitro surrogate or facsimile model). For IL-8 or other alpha chemokine biological activity is characterized by its chemotactic activity (preferably PMNs, but may also include T lymphocytes and/or
25 monocytes/macrophages).

Chemokine: A biological molecule capable of attracting a subset of cell population from the circulating blood to the site of its presence in a gradient dependent fashion

30 PMN: Polymorpha-nuclear neutrophils represent the mature form of circulating leukocyte population that has evolved from the granulocytic lineage and that goes

through the developmental stages of myeloblast, promyelocyte, myelocyte and metamyelocyte. PMNs primarily refer to neutrophils. Other cell types include the monocyte, which also comes from the same granulocyte lineage and evolves from promonocyte to monocyte.

- 5 Assay or Screen: A method used to evaluate the efficacy (agonism or antagonism) of the chemical compounds or biological factors in a given assay system. The system may be amenable for high throughput efficiency.

Modulate: An increase or decrease seen in a set pattern of activity in a system upon addition or deletion of another factor in the same system

- 10 The present invention relates to the ability of CSFs to synergize the attractive capabilities of chemokines to leukocytes, preferably PMNs. As discussed in the Example below, G-CSF appears to be critical for neutrophil recruitment and exhibits synergizing activity with IL-8. In a preferred embodiment of the present invention, antagonism of G-CSF receptor activity would potentially be useful in inhibiting or hindering the inflammatory diseases involving neutrophil exudates, such as sepsis. The present invention, therefore, provides an antagonist of CSF capable of inhibiting or minimizing the attractive capabilities of chemokines for leukocytes and of inhibiting or minimizing leukocyte accumulation and/or activation in vitro and in vivo.

- 20 Chemokines may be identified by any one or more of the characteristics set out above, in particular by their ability to attract and/or activate PMNs, monocytes or lymphocytes in vitro and cause their accumulation and/or activation in vivo. A characteristic that assists the identification of a molecule as a chemokine is its attractive effect on neutrophils or monocytes the other predominant cell type in the circulating blood.

25

The present invention provides a method of determining the ability of a substance to inhibit PMN or monocyte accumulation and/or activation *in vivo*, that is to say, a method for testing putative CSF antagonists, which comprises administering the substance, generally intradermally, to a test animal previously treated with labelled, for example

neutrophils, and subsequently determining the number of labelled neutrophils at a skin site. Other general methods for determining chemotactic activity in vitro may be used to test putative CSF antagonists in vitro.

5 Leukocytes, such as neutrophils, monocytes, basophils, and eosinophils contain an armory of chemicals necessary for killing parasites. These chemicals have been implicated in the damage to airway epithelium that occurs in asthma and may relate to the observed changes in airway function. The studies discussed above and presented herein suggest that CSFs should be considered as important mediators of leukocyte
10 accumulation in vivo. Macrophages, lymphocytes, neutrophils, mast cells, airway epithelial cells, connective tissue cells, vascular endothelial cells, and eosinophils are likely candidates as the source of the PMN chemoattractant activity generated in the lung. Platelets may also have a role as it has been shown that they can release C-C chemokines. Further, an early platelet deposition may be involved in the subsequent PMN
15 accumulation in vivo and there is evidence that platelet-activating factor induces the synthesis of an unidentified PMN chemoattractant in vivo. In this respect, it is of interest that platelet-derived growth factor can induce gene expression of C-C chemokines in fibroblasts. Furthermore, the C-C chemokines have been implicated in wound healing). This may be important in the sub-epithelial basement membrane fibrosis that is a
20 prominent feature of the asthmatic lung. Thus, CSFs may be involved in both PMN accumulation and in chronic structural changes in the lung.

CSF antagonists may have an important role in asthma and in other diseases having an inflammatory component where leukocyte accumulation and/or activation is a prominent
25 feature, for example, atherosclerosis, arthritis, rhinitis and eczema, especially allergic eczema. Accordingly, agents that inhibit or otherwise hinder the production, release or action of CSFs have potential as selective therapeutic agents. Such agents and their therapeutic use are part of the present invention.

30 Such agents include inhibitors that affect the interaction of a CSF with CSF receptors, for example, by binding to a CSF or to a CSF receptor, such as those CSF receptors that

affect a chemokine's attractive abilities to leukocytes. An example of such an inhibitor is receptors themselves which, on administration, can bind a CSF and prevent its interaction with naturally-occurring receptors. Such inhibitory receptors may be soluble or insoluble. Receptors which are not involved in cell activation may be bound to, or induced on, cells.

- 5 Such receptors may also be used to remove endogenous CSF.

Further examples of agents that affect the interaction of CSF with CSF receptors are receptor antagonists, and antibodies, both antibodies directed against (capable of binding with) a CSF and antibodies directed against a CSF receptor, especially monoclonal antibodies. Any other agent that inhibits or otherwise hinders the binding of a CSF to a CSF receptor also has therapeutic potential, for example, any other agent that binds to a CSF or to a CSF receptor. Further agents that have therapeutic potential are those that prevent or reduce activation of CSF receptors.

- 10
15 Further agents that inhibit or otherwise hinder the action of CSFs are those that change the structure of a CSF such that it is no longer able to bind to a CSF receptor, for example, an enzyme or other agent that degrades CSF specifically.

- 20 Receptor promiscuity is common among CSFs, so although it is essential that a receptor is capable of binding a CSF, the receptor need not necessarily be G-CSF-specific. For example, a receptor may bind GM-CSF , M-CSF and/or other leukocyte attractant CSF as well as a G-CSF.

- 25 As indicated above, possibilities for therapeutic intervention include the use of a receptor to which a CSF binds, especially a soluble receptor. It may be advantageous to use a CSF-specific receptor. Further possibilities for therapeutic intervention include receptor antagonists, for example, based on 3-dimensional structures or the amino acid sequences of CSFs and/or of CSF receptors, and agents found to inhibit CSF or other agonists binding to or activating CSF receptors

Agents that prevent or inhibit CSF synthesis or release may also be used therapeutically. Such agents and their use are also part of the present invention.

All inhibitors of CSF activity synthesis and release, including soluble receptors, antibodies, antagonists and inhibitors of agonist binding, and their use are part of the present invention.

The present invention accordingly provides an agent that inhibits or otherwise hinders the production, release or action of a CSF, especially an agent as described above, for use as a medicament. The invention also provides the use of an agent that inhibits or otherwise hinders the production, release or action of a CSF, especially an agent as described above, in the manufacture of a medicament for the treatment of asthma or another disease having an inflammatory component, particularly with accumulation of neutrophils, for example in ischemia reperfusion or acute respiratory distress or eosinophils, for example, rhinitis or eczema, especially allergic eczema syndrome.

Putative inhibitors of CSF activity may be screened using in vivo and in vitro assays based on inhibition of chemoattraction and/or accumulation and/or activation of leukocytes by CSFs. Some general methods for testing the activity of a compound for an inhibitory effect on the activity of a chemoattractant chemokine in vitro are known. Such assays may be used to determine the inhibitory action of a putative inhibitor on in vitro effects induced in leukocytes by the synergistic activity of CSFs on chemokines.

Examples of in vitro and in vivo assays both for the determination of CSF activity and for the determination of CSF inhibitory activity are described herein. For example, Example 1 gives a detailed protocol for the in vitro assay of the present invention. The assays described herein may be used as such, or may be modified as required. Assays may be used alone or in combination with other assays known to those skilled in the art to establish CSF and CSF-inhibitory activity. A putative inhibitor may be any of the types of molecules described above, including receptors, for example, soluble receptors, antibodies, and antagonists and inhibitors of agonist binding. Methods for testing putative inhibitors of CSFs are also part of the present invention. In a preferred embodiment of

the present invention, the protocol screening assay for G-CSF receptor antagonists is as described in Example 2.

A further aspect of the present invention is a pharmaceutical preparation comprising, as
5 active ingredient, an agent that inhibits or otherwise hinders the production, release or action of a CSF, in admixture or in conjunction with a pharmaceutically suitable carrier. Such agents are described above and include, for example, an inhibitor of CSF synthesis or release, a soluble CSF receptor, a CSF receptor antagonist or an inhibitor of a CSF receptor agonist, an antibody against CSF or an antibody against a CSF receptor.

10 The invention further provides a method of treating asthma, ischemia reperfusion injury and other inflammatory diseases, comprising the administration of an effective amount of an agent that inhibits or otherwise hinders the production, release or action of a CSF. The agent may be as described above, for example, an inhibitor of CSF synthesis or release, a
15 soluble CSF receptor, a CSF receptor antagonist or an inhibitor of a CSF receptor agonist, or an antibody against CSF or against a CSF receptor.

The present invention also provides assays for antagonists of CSF and for anti-CSF antibodies, especially immunoassays and in particular ELISAs (enzyme-linked
20 immunosorbent assays). The invention provides, for example, an immunoassay for an antigen, characterized in that the antigen is a CSF, and also provides an immunoassay for an antibody, characterized in that the antibody is an anti-CSF antibody. The invention also provides assays for CSFs that are analogous to immunoassays for CSFs but that use a specific-binding partner other than an antibody. In such specific-binding partner assays
25 a CSF receptor may be used instead of an anti-CSF antibody.

In an immunoassay, an anti-CSF antibody may, for example, be coated on a solid surface to enable capture and hence detection of CSF. An anti-CSF antibody may be used in an assay for the detection of antibodies to CSF, for example, in a competitive antibody
30 assay. A labeled CSF or a derivative thereof, for example, a recombinant CSF or a synthetic peptide comprising part of the amino acid sequence of an CSF may be used in a

competitive antigen assay for CSF or may be used to coat a solid surface in a capture assay for antibodies to CSF. The many different types of assay format are well described in the literature of the art, see for example "ELISA and other Solid Phase Immunoassays, Theoretical and Practical Aspects" Eds. Kemeny D. M. & Challacombe S. J., John Wiley, 1988. (36). Assays using an CSF receptor instead of an anti-CSF antibody may be carried out analogously.

The preferred use of the CSF antagonizing compounds of the invention is for, but not limited to, the treatment of chronic and acute inflammatory and auto-immune diseases such as SLE, GVHD, RA, IBD, asthma and Psoriasis.

The dose and dosage regiment of a CSF antagonizing compound according to the invention that is suitable for administration to a particular patient can be determined by a physician considering the patient's age, sex, weight, general medical condition, and the specific condition and severity thereof for which the CSF antagonizing compound is being administered; the route of administration of the CSF antagonizing compound; the pharmaceutical carrier with which the CSF antagonizing compound may be combined; and the CSF antagonizing compound's biological activity.

Generally, intravenous subcutaneous or transmuscular injection of 1-500 μ mol of CSF antagonizing compounds/kg body weight, by bolus injection, by infusion over a period of about 5 minutes to about 60 minutes, or by continuous infusion is sufficient for therapeutic efficacy. Aerosol inhalation of 0.1 or 2 mg of CSF antagonizing compounds/kg body weight is also sufficient for efficacy.

Intravenous, subcutaneous or intramuscular administration, by bolus injection or continuous infusion, is preferred for use of the analogs of the invention in treatment of autoimmune or inflammatory disease.

The CSF antagonizing compounds of the invention, or a pharmaceutically acceptable salt thereof, can be combined, over a wide concentration range (e.g., 0.001 to 11.0 wt %) with

any standard pharmaceutical carrier (e.g., physiological saline, THAM solution, or the like) to facilitate administration by any of various routes including intravenous, subcutaneous, intramuscular, oral, or intranasal, including by inhalation.

- 5 Pharmaceutically acceptable acid addition salts of the CSF antagonizing compounds of the invention can be prepared with any of a variety of inorganic or organic acids, such as for example, sulfuric, phosphoric, hydrochloric, hydrobromic, nitric, citric, succinic, acetic, benzoic and ascorbic. The analogs can, for example, be advantageously converted to the acetate salt by dissolution in an aqueous acetic acid solution (e.g., 10% solution)
10 followed by lyophilization.

Pharmaceutical compositions containing a CSF antagonizing compound of the present invention as the active ingredient in intimate admixture with a pharmaceutical carrier can be prepared according to conventional pharmaceutical compounding techniques. The
15 carrier may take a wide variety of forms depending on the form of preparation desired for administration, e.g., intravenous, oral or parenteral. In preparing the compositions in oral dosage form, any of the usual pharmaceutical media may be employed, such as, for example, water, glycols, oils, alcohols, flavoring agents, preservatives, coloring agents and the like in the case of oral liquid preparations (such as, for example, suspensions,
20 elixirs and solutions); or carriers such as starches, sugars, diluents, granulating agents, lubricants, binders, disintegrating agents and the like in the case of oral solid preparations (such as, for example, powders, capsules and tablets). Because of their ease in administration, tablets and capsules represent the most advantageous oral dosage unit form in which case solid Pharmaceutical carriers are obviously employed. If desired,
25 tablets may be sugar-coated or enteric-coated by standard techniques. For parenterals, the carrier will usually comprise sterile water, though other ingredients, for example, to aid solubility or for preservative purposes, may be included. Injectable suspensions may also be prepared, in which case appropriate liquid carriers, suspending agents and the like may be employed. The pharmaceutical compositions will generally contain dosage unit, e.g.,
30 tablet, capsule, powder, injection, teaspoonful and the like, from about 0.001 to about 10 mg/kg, and preferably from about 0.01 to about 0.1 mg/kg of the active ingredient.

5

Materials and Methods

10

20

30

In Vivo Recruitment Assay: At t=0, rabbits received 14.5 ng/kg (10 μ Ci) 125 I- IL-8 *i.v.* At t=15 min rabbits were anesthetized with isoflurane and injected intradermally (100 μ L/site) with 0.01-3.3 μ g IL -8 or IL-8 and G-CSF (100 pM). The vehicle consisted of sterile saline supplemented with 0.2% bovine serum albumin and 0.01 mM PGE₂ and 0.1 μ M bradykinin. At t=75 min, rabbits were euthanized with 1 mL *i.v.* dose of Beuthanasia-D Special (Shering-Plough Animal Health, Kenilworth, NJ) and skin biopsies were obtained using a 6.35 mm diameter punch (O'Brien Consolidated Industries, Lewiston, ME). The biopsies were weighed and the radioactivity was determined using a gamma counter (Packard Model #05005, Downers Grove, IL).

Calcium Flux Assay: Calcium flux in response to IL-8 with or without G-CSF was assessed using standard fluorescent emission protocol. Briefly, neutrophils were loaded with 4 μ M of Fluo-4, for one hour at 37°C. In a 96 well plate approximately 300,000 cells/well were preincubated with or without G-CSF (0.1 pM to 1000 pM) for the respective time periods indicated and then were subsequently stimulated with IL-8. Intracellular calcium flux in the cell was measured using FLIPR³⁸⁴ (Molecular Devices, Sunnyvale, CA).

Binding Assay: The IL-8 and G-CSF binding assays were performed on the isolated human neutrophils using 0.3 ml micro sedimentation tubes (Sarstedt, Newton, NC). A small amount (9.5 μ l) of sucrose solution (0.4%) was placed through the narrow tip of the tube by centrifugation. The reaction mix was layered gently on the sucrose solution to allow an air bubble in between the two solutions. In IL-8 binding studies, a total of 40,000 cells with 0.5 nM of hot IL-8 were used in each reaction. In cold competition studies, excess of 500 fold cold IL-8 was added. To quantitate the G-CSF binding on PMN, the cell number was varied from 50,000 to 1,000,000 per reaction with 5nM of 125 I- G-CSF. In both binding studies, the cells were incubated with hot ligand for 3 hours and were pelleted at 10,000 RPM for 2 min. The sucrose layer was used to separate the bound from the unbound radio-labeled ligand. The cell pellet was cut out with a razor blade and the radioactive counts in the cell pellet were measured using a gamma counter.

Several experiments were conducted to confirm that G-CSF synergizes the IL-8 induced chemotaxis in vivo and in vitro. The methods and materials employed to conduct the studies are discussed in detail below and serve to guide those skilled in the art in understanding the inventions described herein. The synergy of G-CSF on IL-8

5 chemotaxis is demonstrated in Figure 1 where increases in G-CSF concentration, on a picomolar level, increases the chemotactic response of neutrophils. Similarly the synergy of GM-CSF on IL-8 chemotaxis is demonstrated in Figure 2. Figure 3 illustrates the dose response curve for IL-8 with fixed G-CSF concentration and demonstrates a ten fold increase in neutrophil response to a combination of IL-8 and G-CSF. Figure 4 shows
10 that G-CSF does not synergize the f-MLP induced neutrophil chemotaxis, indicating an IL-8, and chemokines functionally and/or structurally similar to IL-8, specific phenomenon. Figure 5 shows that the synergy is not limited to in vitro studies, but is also shows that G-CSF enhances in vivo neutrophil intradermal recruitment. Figure 6 illustrates and confirms the binding of I^{125} G-CSF to neutrophils. Figure 7 illustrates that
15 G-CSF neutralizing antibody inhibits G-CSF synergized chemotaxis. Figure 8 illustrates that G-CSF pre-incubation alters PMN chemotactic response to IL-8. Figures 9 and 10 demonstrate that G-CSF synergism is independent of its effects on IL-8 binding or IL-8 induced calcium flux, suggesting involvement of a signaling mechanism. The study was conducted by employing radiolabeled I^{125} G-CSF and studying its effects on polymorpho
20 nuclear neutrophils (PMN). Figure 11 illustrates that G-CSF pre-incubation does not alter IL-8 binding. Figure 12 illustrates that G-CSF pre-incubation alters PMN response to IL-8.

G-CSF was traditionally known to be a hematopoietic growth factor and also known to
25 enhance functional capacities of the neutrophils through maturation of the cell. However, surprisingly, in G-CSF knock out mice, the circulating mature neutrophil number was less, but maturation was not defective, attributing additional roles for the traditionally known hematopoietic growth factor. Similar observations were made in G-CSF receptor knock out mice. In addition, it was observed that the neutrophils from G-CSF receptor
30 knockout mice do not chemotax in response to IL-8 or f-MLP. Based on these studies, we proposed that a synergism exists between G-CSF and other chemokines for its

functional activation, in vivo. Our studies, in vivo and in vitro support the synergistic mechanisms that exist between these molecules. In vivo, in inflammatory situations, picomolar concentrations of G-CSF are found at the site along with nanomolar concentrations of chemokines. Hence, it is quite possible that the synergistic mechanisms that we found here in the study are biologically significant. Hence, blocking the synergism using strategies that antagonize G-CSF or the receptor to prevent inflammation would be a useful tool to prevent the inflammatory process

Example 2

10 Protocol for a potential screening assay for G-CSF receptor antagonists:

This assay utilizes the binding properties of G-CSF directly to its receptor on the cells.

1. Obtain G-CSF receptor cDNA from the human genomic DNA synthesized from human neutrophils. The cDNA can be obtained by polymerase chain reaction (PCR) using primers aligned to the 5' and 3' ends of the mRNA sequence that is readily available in the GeneBank.
2. Clone the G-CSFR cDNA into a plasmid such as pcDNA 3.1 and stably transfect into a hematopoietic cell line that closely resembles the circulating leukocytes. Stably integrated clones can be screened by using a antibiotic resistance marker such as gentamycin (G418)
3. Using radiolabeled G-CSF the expression of this transfected G-CSFR can be quantitated and the binding of G-CSF can be measured. Briefly, the binding is performed using radiolabeled (¹²⁵I) G-CSF. This is incubated with the stably transfected cells in a tube for 3 hours in the presence or absence of the cold unlabeled ligand. After three hours, the unbound fraction of the hot ligand will be separated using sucrose gradient separation technique. The cells with the bound hot ligand are pelleted and the amount of incorporated radioactive material will be measured using the scintillation counter. The amount of scintillation count represents the amount of binding and a good ratio between the hot and hot+cold binding represents the less

non-specific binding. Based on the binding, further selection for high expressing clones will be performed.

4. To screen for G-CSFR antagonists, the cells are first pre-incubated with the chemical compound(s) for 30 minutes at which point, the hot radiolabeled G-CSF is added and further incubated for 3 hours more. An antagonist will decrease the binding of the G-CSF to its receptor and will be evident from the decreased binding on the cells.
5. The effective concentrations of the antagonists will be evaluated by performing a dose-response curve on these compounds. An antagonist might represent a molecule that binds to the receptor and does not allow the ligand to bind or it could be a compound that binds to the ligand and does not let the ligand bind to the receptor.

Similar protocols can be developed to screen for G-CSF antagonists using a kinase assay systems or gene transcriptional activation systems using reporter constructs. All these protocols are amenable for the high throughput screening mode.

CLAIMS

What is claimed is:

1. An antagonist of a CSF capable of antagonizing the the attracting capabilities of chemokines and of inducing the accumulation and/or activation in vitro and in vivo of key components of inflammatory responses.
2. The antagonist of claim 1 wherein the CSF is G-CSF.
3. The antagonist of claim 1 wherein the CSF is M-CSF.
4. The antagonist of claim 1 wherein the CSF is GM-CSF.
5. The antagonist of claim 1 wherein the chemokine is an alpha chemokine.
6. The antagonist of claim 1 wherein the chemokine is a beta chemokine.
7. The antagonist of claim 1 wherein the component of inflammatory response is a neutrophil.
8. The antagonist of claim 1 wherein the component of inflammatory response is a monocyte.
9. A method for treating inflammatory disorders and diseases comprising the step of administering to a mammal in need thereof a therapeutically effective amount of an antagonist of a CSF capable of synergizing the attracting capabilities of chemokines and of inducing the accumulation and/or activation in vitro and in vivo of key components of inflammatory responses.
10. The method of claim 9 wherein the inflammatory disorder or disease is sepsis.
11. The method of claim 9 wherein the inflammatory disorder or disease is asthma.
12. The method of claim 9 wherein the inflammatory disorder or disease is an autoimmune disease.
13. A pharmaceutical composition comprising said antagonist of claim 1 combined with a pharmaceutically acceptable carrier.
14. The method of claim 9 wherein the inflammatory disorder or disease is a chronic inflammatory disorder or disease.
15. A method for screening for an antagonist of a CSF capable of synergizing the attracting capabilities of chemokines and of inducing the accumulation and/or

5

ABSTRACT

5

Fig. 1. G-CSF Synergizes IL-8 Induced Neutrophil Chemotaxis

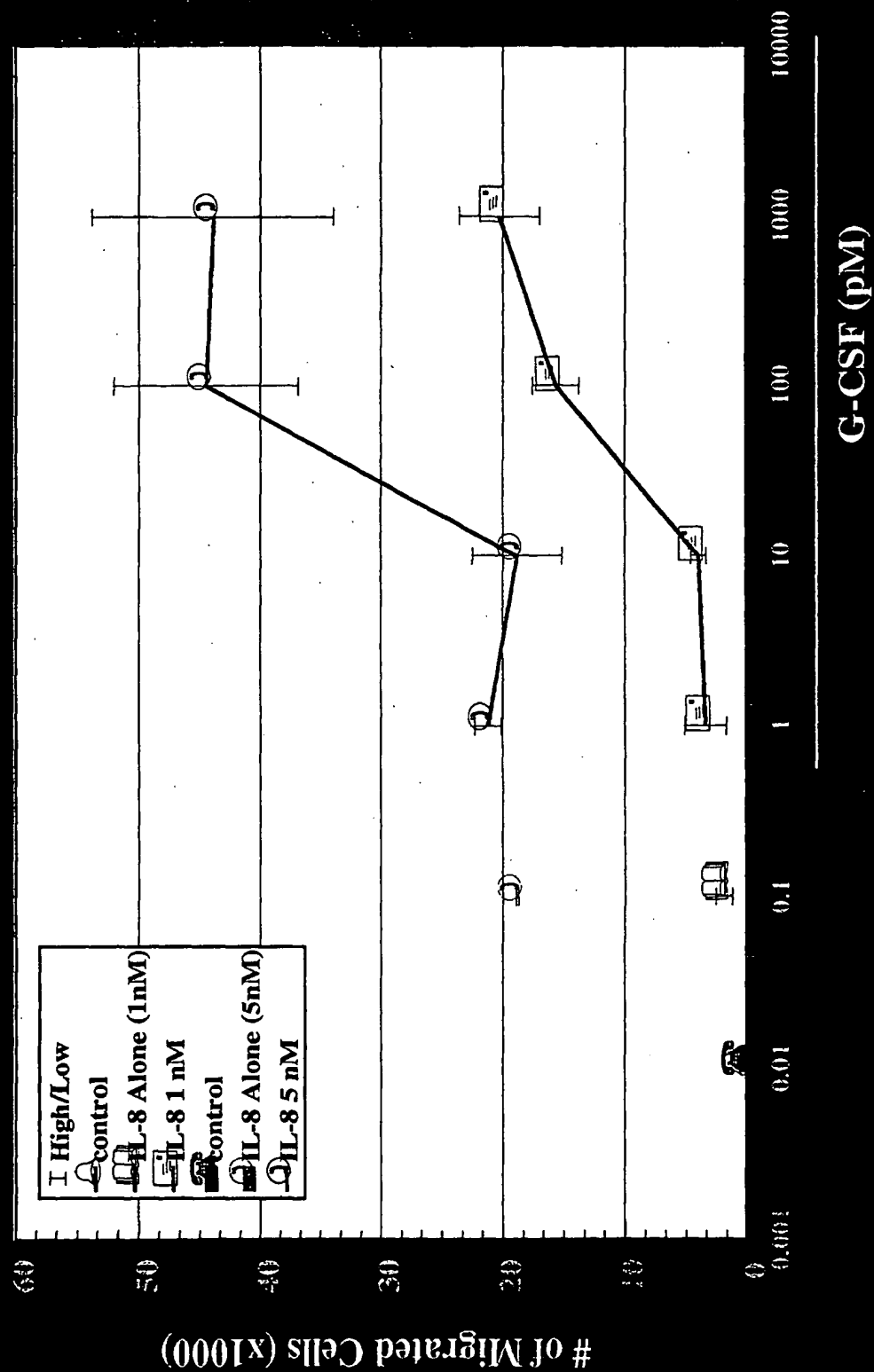


Figure 2
GM-CSF Synergizes IL8 Induced PMN Chemotaxis

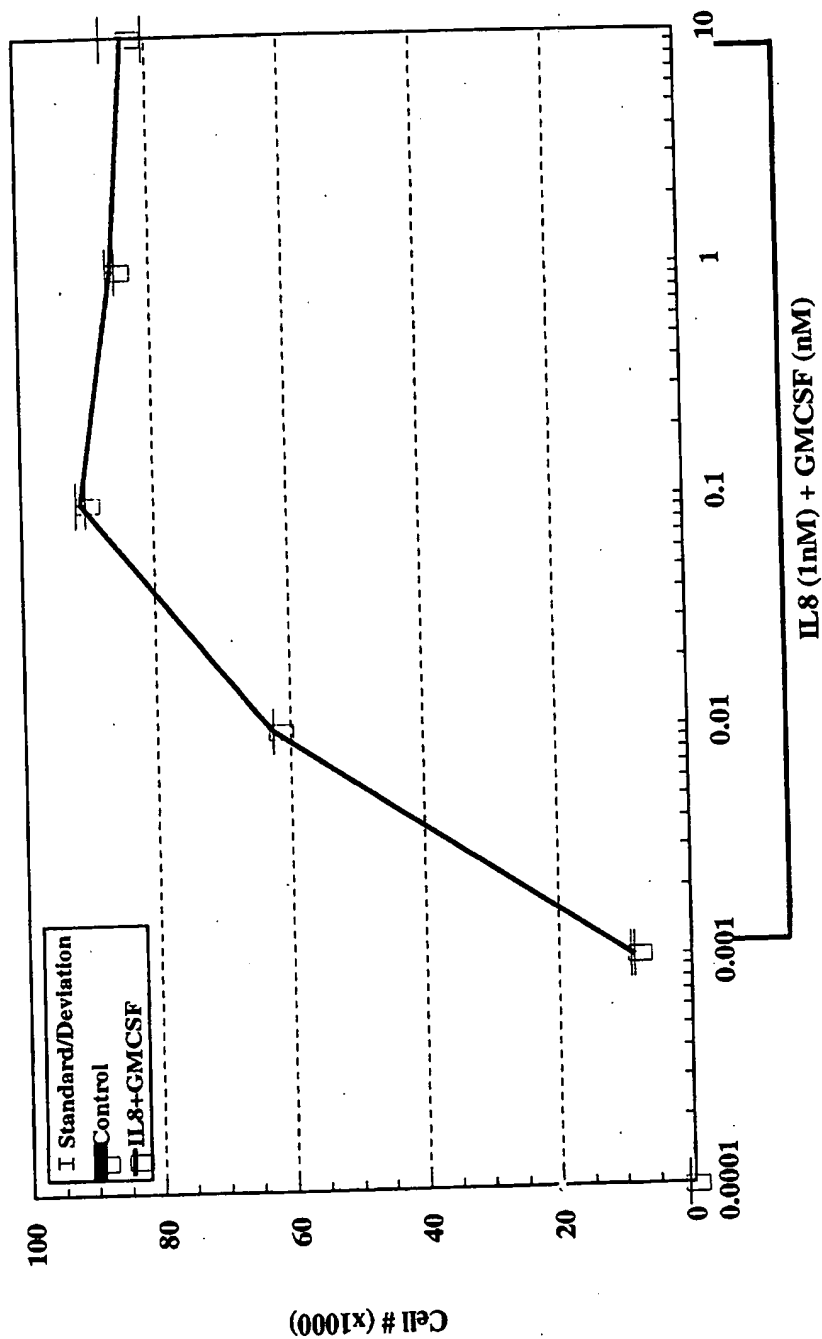


Fig. 3. Dose Response Curve for IL-8 with Constant G-CSF (100 pM)

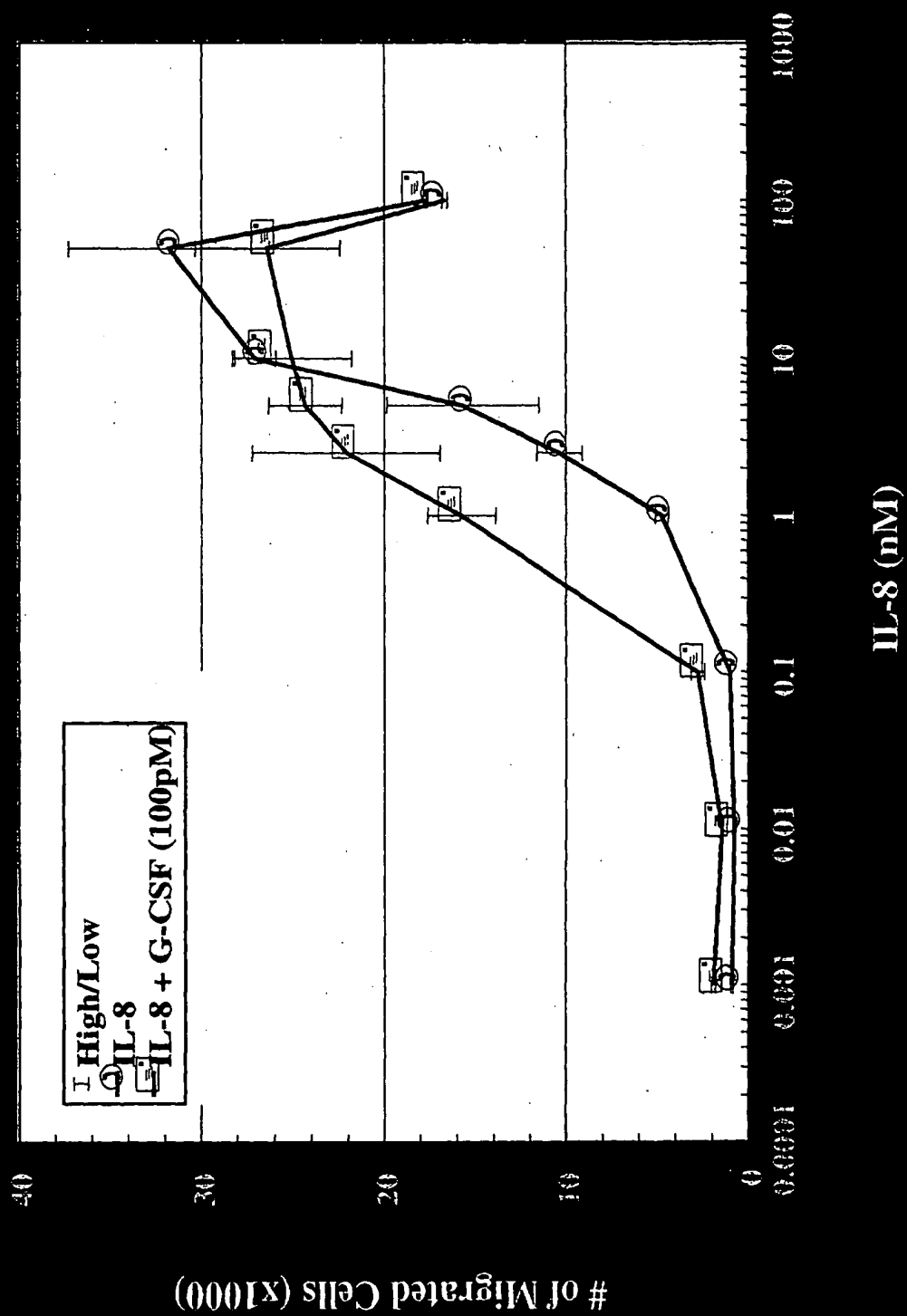


Fig. 4. GCSF Does not Synergize f-MLP
Induced Neutrophil Chemotaxis

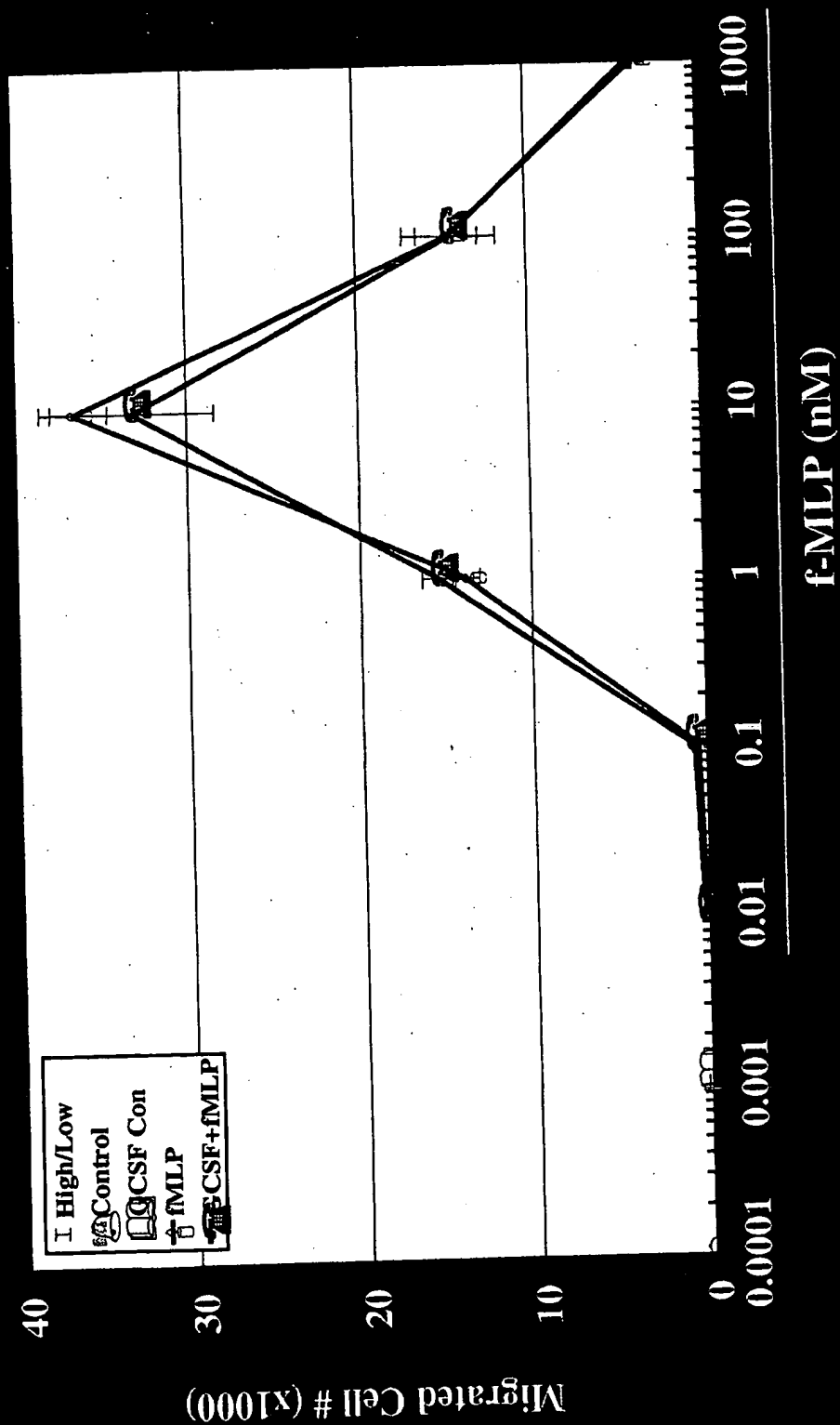


Fig.5. G-CSF enhances *in vivo* neutrophil intradermal recruitment

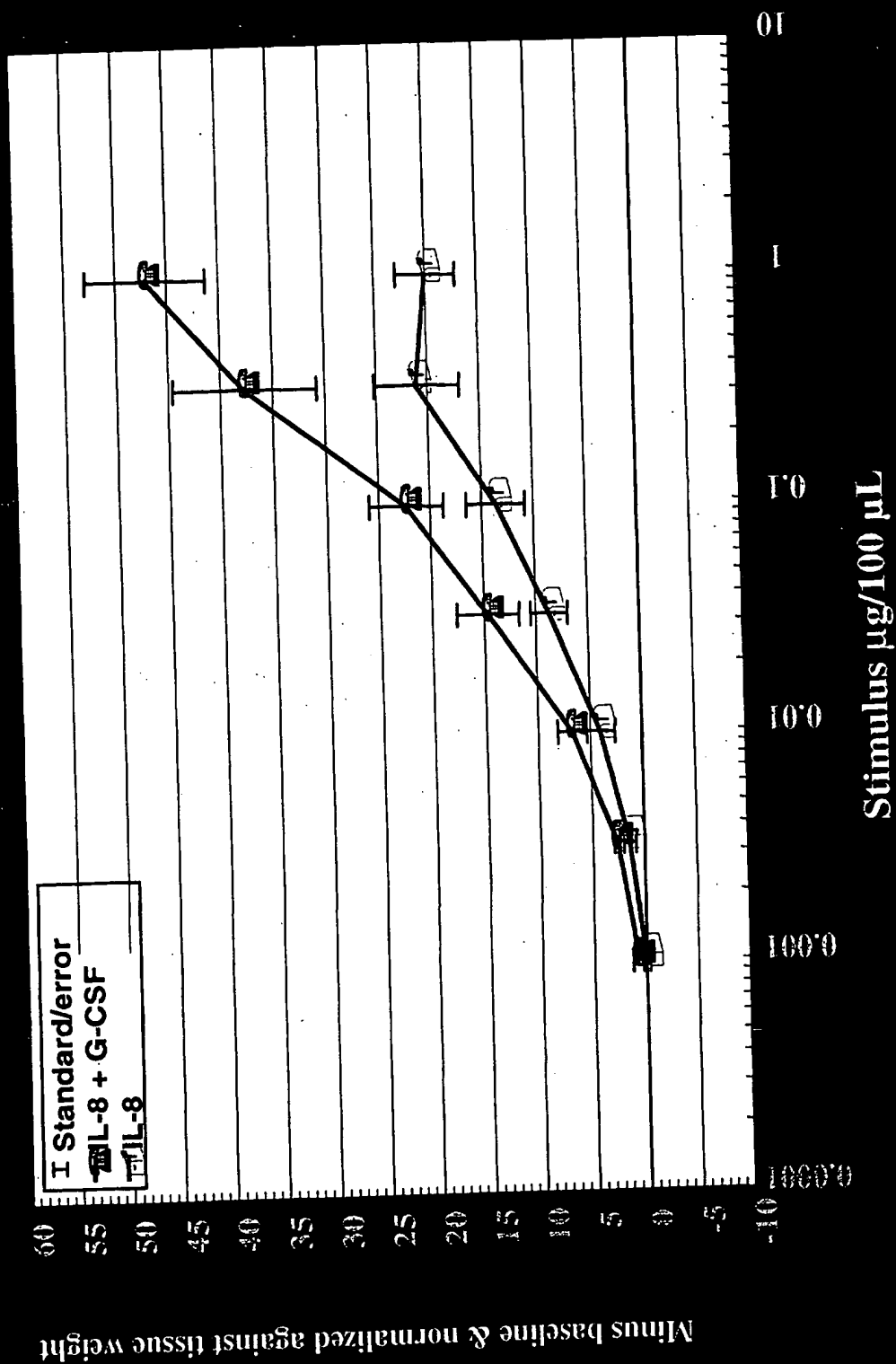


Fig. 6. Binding of ^{125}I G-CSF on PMN

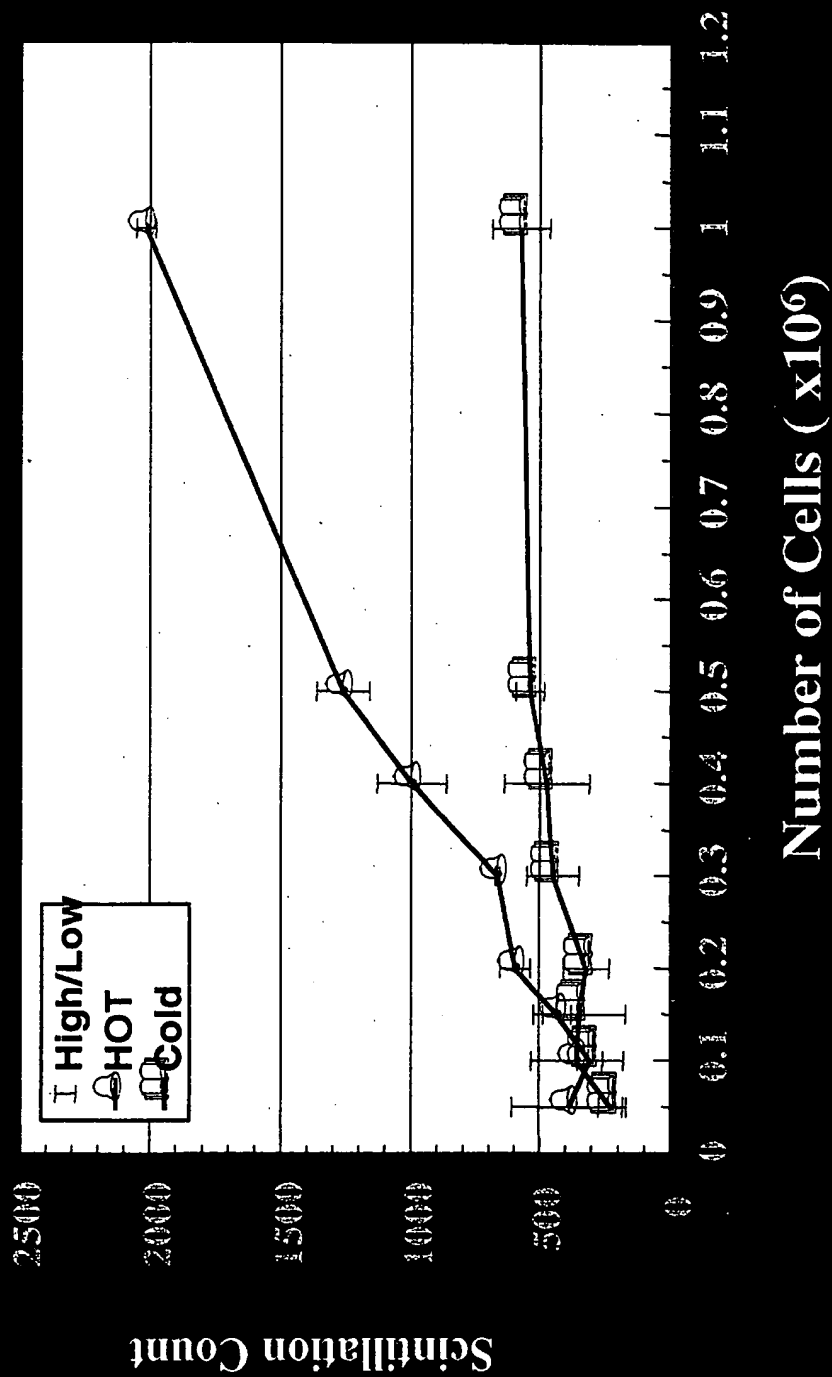


Fig. 7. G-CSF Neutralizing Antibody Inhibits
G-CSF Synergized Chemotaxis

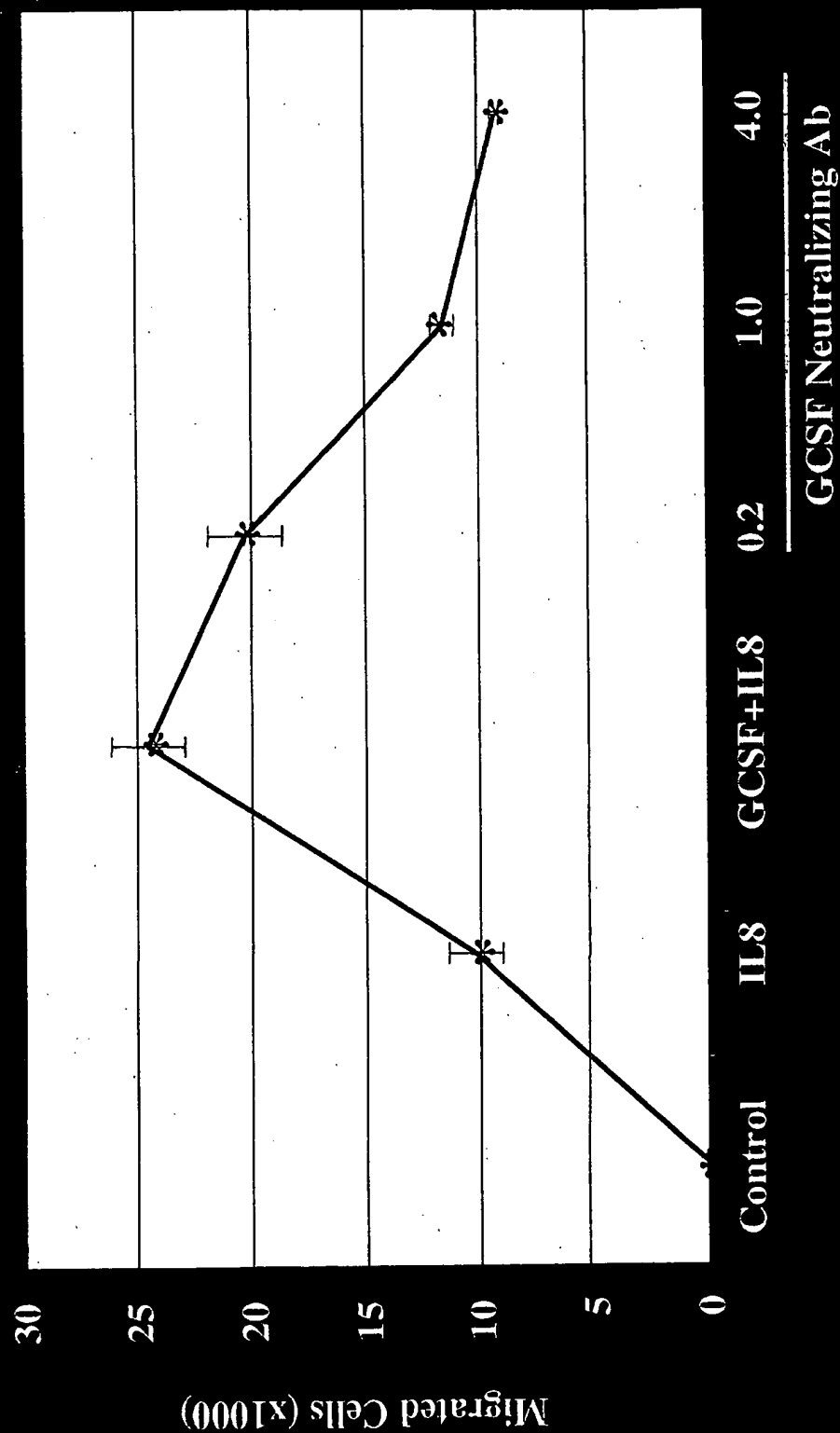
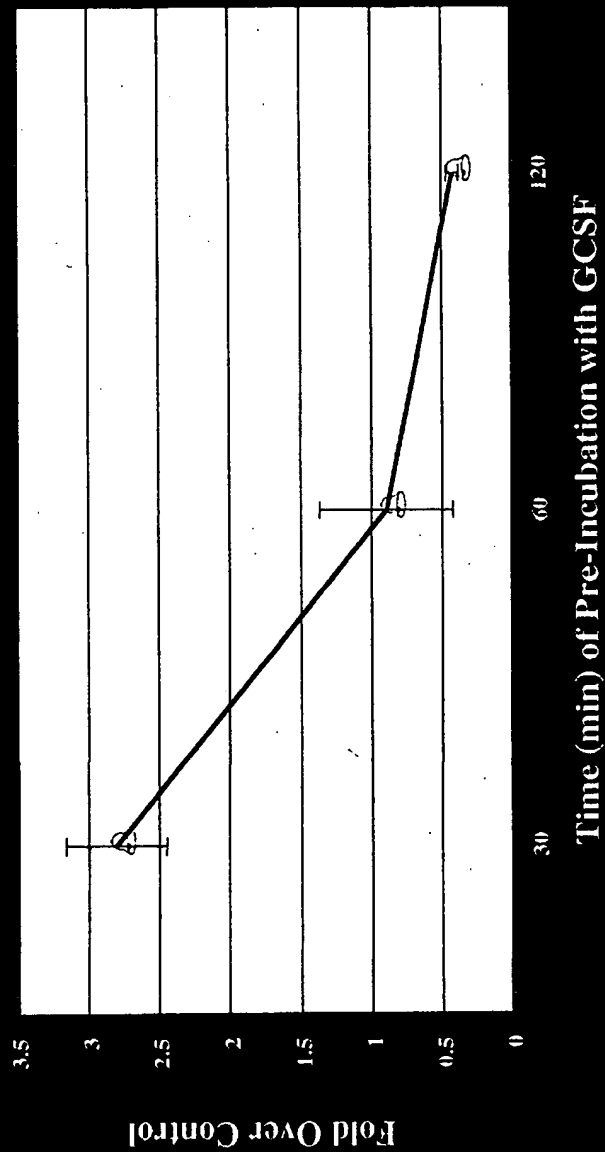


Fig. 3. G-CSF Pre-Incubation Decreases
Neutrophil Response to IL-8



Cells were preincubated with G-CSF for respective time periods and subsequently treated with 1nM of IL-8

Fig. 9. G-CSF Does not Alter IL-8 Induced Calcium Flux

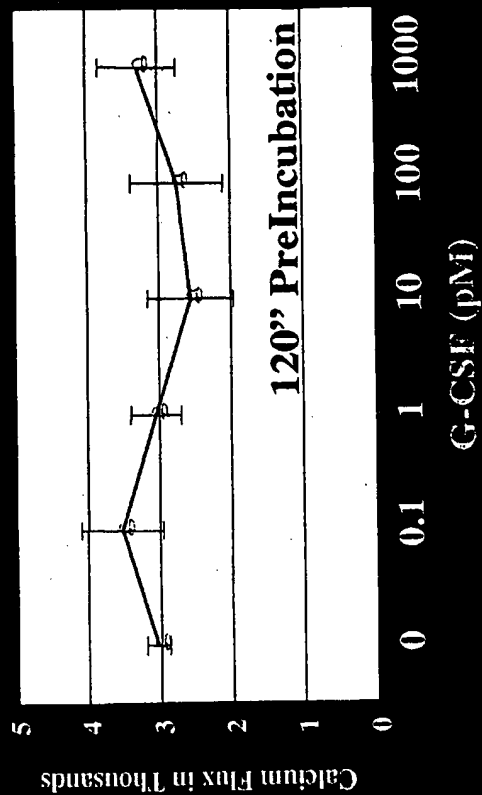
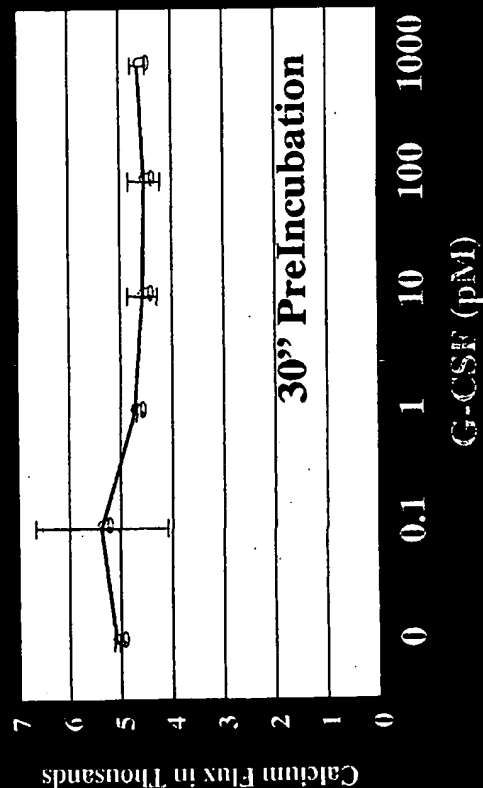
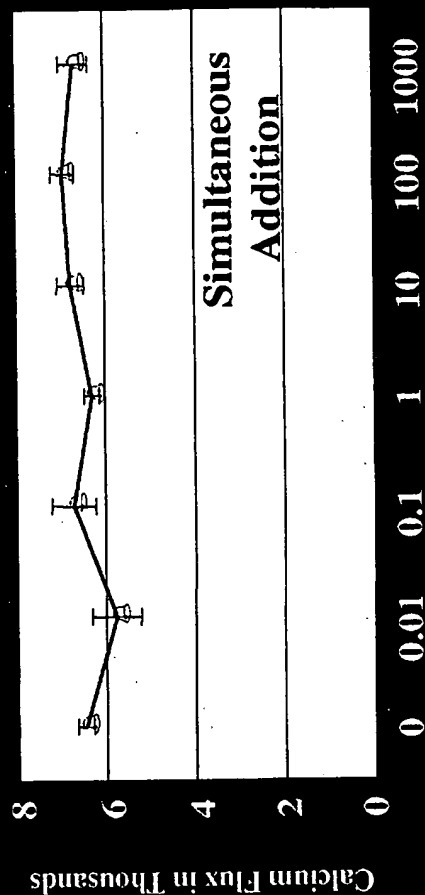


Fig. 10. G-CSF Does Not Increase IL-8 Binding in Neutrophils

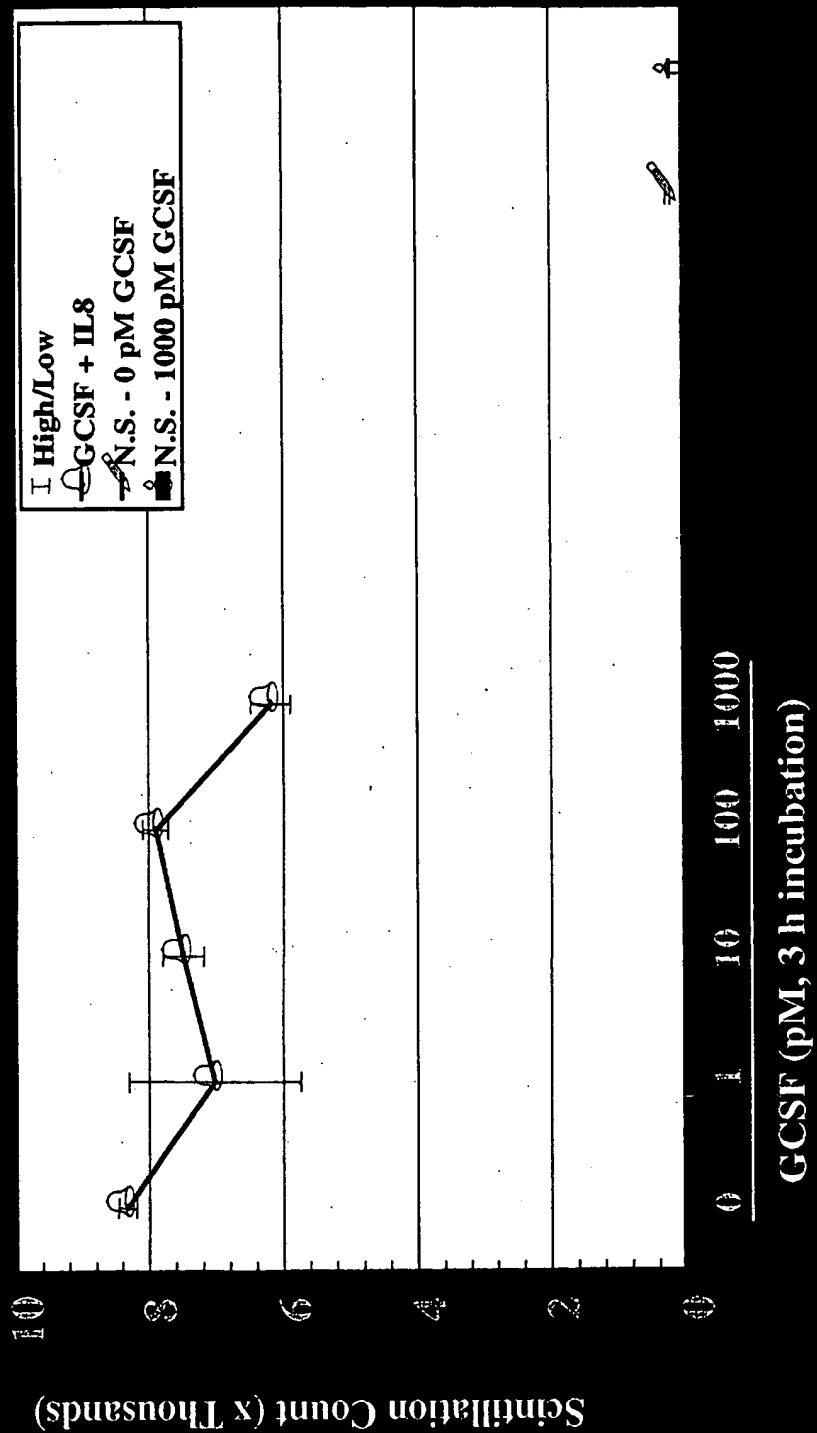
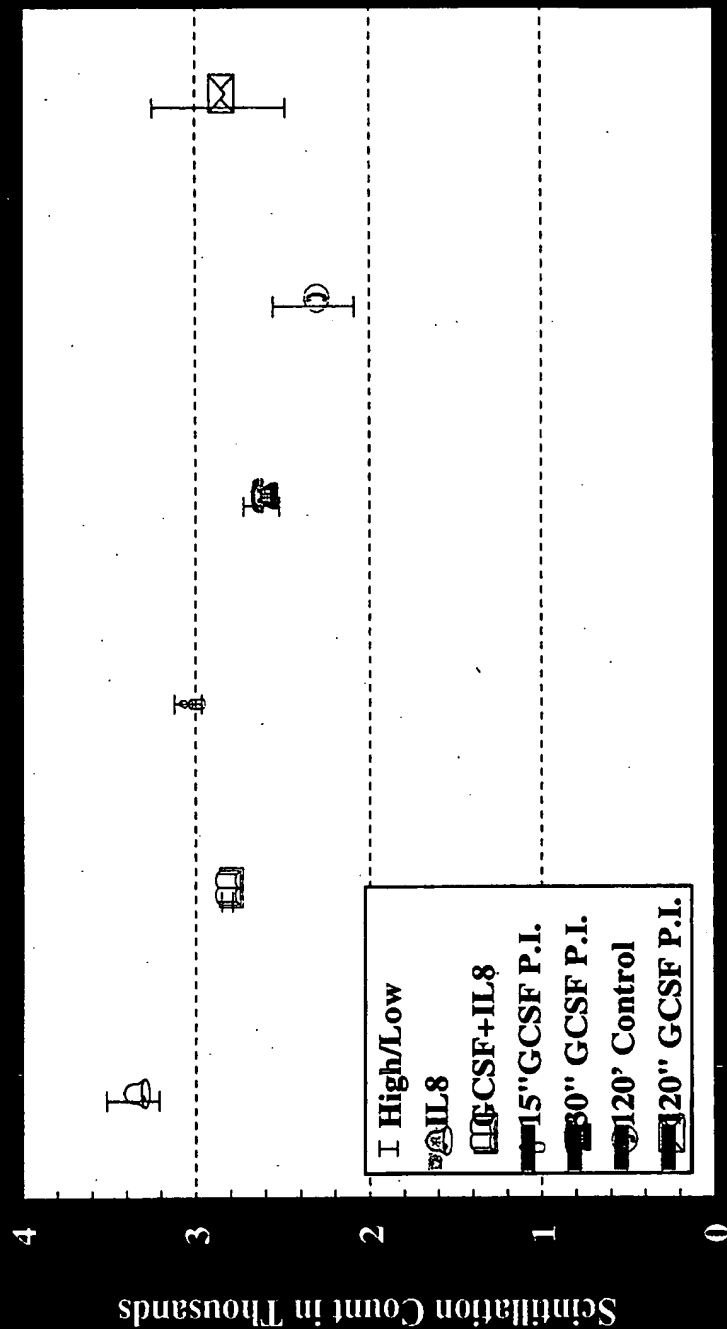


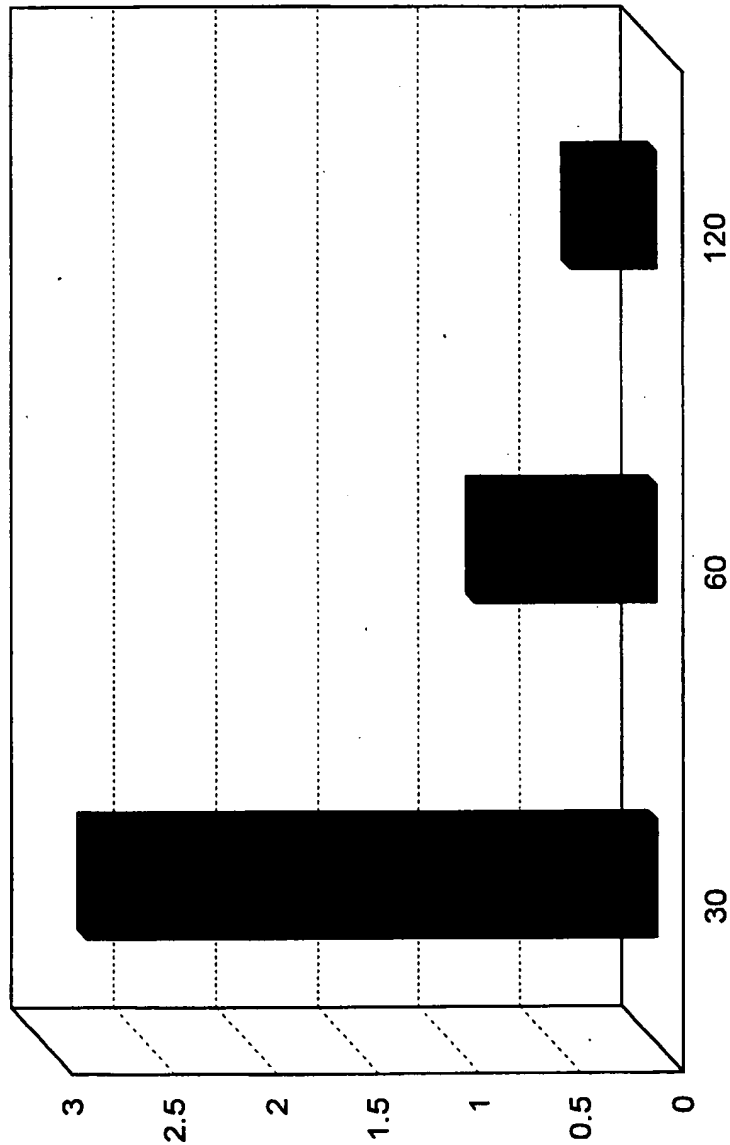
Fig. 11. G-CSF Preincubation Does not Alter IL-8 Binding on Neutrophils



100 pM of G-CSF was incubated simultaneously or pretreated for the respective time periods

FIGURE 12

G-CSF Pre-Incubation Alters PMN Response to IL-8



02-26-01

09885259 .022A002C

Please type a plus sign (+) inside this box



Ticket Number:

A0000326L2-01CFP

PROVISIONAL APPLICATION FOR PATENT COVER SHEET (Large Entity)

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53 (c).

INVENTOR(S)/APPLICANT(S)

Given Name (first and middle (if any))	Family Name or Surname	Residence (City and either State or Foreign Country)
Madhav Narasimha Joseph Edwin	Devalaraja Low	2715 Windwood Dr. #62, Ann Arbor, MI 48105 921 Alpine Court, Brighton, MI 48116

☐ Additional inventors are being named on page 2 attached hereto**TITLE OF THE INVENTION (280 characters max)**

INHIBITORS OF COLONY STIMULATING FACTORS

CORRESPONDENCE ADDRESS

Direct all correspondence to:

☐ Customer Number Place Customer Number
Bar Code Label here

OR

<input checked="" type="checkbox"/> Firm or Individual Name	Claude F. Purchase, Jr.				
Address	Warner-Lambert Company				
Address	2800 Plymouth Road				
City	Ann Arbor	State	Michigan	ZIP	48105
Country	USA	Telephone	734-622-1692	Fax	734-622-1553

ENCLOSED APPLICATION PARTS (check all that apply)

<input checked="" type="checkbox"/> Specification	Number of Pages	37	<input checked="" type="checkbox"/> Other (specify)	30 claims on 4 pages abstract on 1 page
<input checked="" type="checkbox"/> Drawing(s)	Number of Sheets	21		

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT (check one)

<input type="checkbox"/> A check or money order is enclosed to cover the filing fees	FILING FEE AMOUNT (\$)
<input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge filing fees or credit any overpayment to Deposit Account Number: 23-0455	
	\$150.00

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☒ No.☐ Yes, the name of the U.S. Government agency and the Government contract number are:

Respectfully submitted,

SIGNATURE Claude F. Purchase, Jr.DATE February 23, 2001TYPED or PRINTED NAME Claude F. Purchase, Jr.REGISTRATION NO. P-47,871
(if appropriate)TELEPHONE 734-622-1692**USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT**

SEND TO: Box Provisional Application, Assistant Commissioner for Patents, Washington, DC 20231

09885259.022301

Packet Number:

A0000326L2-01CFP

PROVISIONAL APPLICATION FOR PATENT COVER SHEET (Large Entity)

INVENTOR(S)/APPLICANT(S)		
Given Name (first and middle [if any])	Family Name or Surname	Residence (city and either State or Foreign Country)

Certificate of Mailing by Express Mail

I certify that this application and enclosed fee is being deposited on February 23, 2001 with the U.S. Postal Service "Express Mail Post Office to Addressee" service under 37 C.F.R. 1.10 and is addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231.

Cindy Malocha

Signature of Person Mailing Correspondence

Cindy Malocha

Typed or Printed Name of Person Mailing Correspondence

EK651207059US

"Express Mail" Mailing Label Number

USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT**SEND TO: Box Provisional Application, Assistant Commissioner for Patents, Washington, DC 20231**

09885259 .022301

Express Mail No. EK651207059US

A0000326L2-01CFP

INHIBITORS OF COLONY STIMULATING FACTORS

-1-

INHIBITORS OF COLONY STIMULATING FACTORS

FIELD OF THE INVENTION

The present invention is directed to inhibitors of haematopoietic factors called colony stimulating factors and methods of treating diseases responsive to inhibition of colony stimulating factors. The present invention is also directed to assays for screening inhibitors of CSF.

BACKGROUND OF THE INVENTION

Colony stimulating factors (CSFs) stimulate the differentiation and/or proliferation of bone marrow cells. CSFs in both human and murine systems have been identified and distinguished according to their activities involving two of the three main classes of leukocytes, namely granulocytes and monocytes. For example, granulocyte-CSF (G-CSF) and macrophage-CSF (M-CSF) stimulate the in vitro formation of neutrophilic granulocyte and macrophage colonies, respectively, while granulocyte-macrophage CSF (GM-CSF) has broader activities and stimulates the formation of both macrophage, neutrophilic, and eosinophilic granulocyte colonies. These CSFs act via their respective receptors, namely G-CSFR, M-CSFR, and GM-CSFR. G-CSFR is expressed on multipotential hematopoietic progenitor cells and cells of myeloid lineage, and is important for regulation of granulopoiesis.

Evidence of the role G-CSF and G-CSFR play in inflammation includes the discovery that G-CSF is frequently found elevated in serum of and at inflammatory sites in patients with infections. The undetectable normal circulating levels of G-CSF (≤ 10 pM) increase in inflammatory conditions to a range of from 100 to 2000 pM. Further, transgenic mice with neutrophils expressing chimeric receptors with extra-cellular G-CSFR and intra-cellular erythropoietin receptor appear to retain their normal hematopoietic function but no longer respond to chemotactic signals. Also, the chemokine interleukin-8 (IL-8) fails to induce chemotaxis of neutrophils from G-CSFR $-/-$ mice (i.e., G-CSFR knockout mice),

-2-

suggesting a specific role for G-CSFR in neutrophil chemotaxis. However, by itself, G-CSF is a relatively weak chemoattractant.

Additionally, M-CSF, also known as colony stimulating factor-1, has been shown to increase blood and tissue macrophage numbers in several species. For example, it is known that M-CSF is produced within the joint in human rheumatoid arthritis, where it has been shown to cause severe exacerbation of the disease. This is consistent with other studies, wherein M-CSF was found to worsen the disease course of experimental disseminated candidiasis, a disease with many of the characteristics of tumor necrosis factor-mediated pathology. M-CSF was also found to stimulate secretion of urokinase plasminogen activator, which plays a role in proteolytic joint destruction. Recently, cDNA encoding the primary growth and differentiation factor for M-CSF has been isolated, sequenced and expressed, and human recombinant M-CSF is now available for experimental studies.

However, CSFs are not the only cytokines involved in inflammation. Also involved are chemokines, which are chemotactic cytokines that are released by a wide variety of cells to attract macrophages, T cells, eosinophils, basophils, and neutrophils to sites of inflammation. There are two classes of chemokines, the members of each class share an organizing primary sequence motif. Alpha chemokines such as IL-8, neutrophil-activating protein-2 (NAP-2), and melanoma growth stimulatory activity protein (MGSA) are chemotactic primarily for neutrophils, whereas beta chemokines such as RANTES (regulation-upon-activation, normal T expressed and secreted), MIP-1 alpha (macrophage inflammatory protein), MIP-1 beta, MCP-1 (monocyte chemotactic protein-1), MCP-2, and MCP-3 are chemotactic for monocytes, T-cells, eosinophils, and basophils.

Chemokines bind specific cell-surface receptors belonging to the family of G-protein-coupled seven-transmembrane-domain proteins which are termed "chemokine receptors." Chemokines and chemokine receptors such as, for example, CCR-1, CCR-2, CCR-2a, CCR-2b, CCR-3, CCR-4, CCR-5, CXCR-1, CXCR-2, CXCR-3, and CXCR-4, play a role in inflammation and autoimmune responses by attracting leukocytes, which migrate out of the microvasculature and into the extravascular space in response to chemoattractant molecules. These

THIS PAGE BLANK (USPTO)

chemoattractants, which include cytokines and activated complement components, may be released by the patient or they may be released from an invading organism. Once exposed to chemoattractants within the vasculature, the leukocytes become activated and capable of adhering to the endothelium, providing the first step in the development of inflammation. Stimulated neutrophils adhere to the endothelium of the microvasculature in response to a gradient of chemoattractants which direct the cells into the extravascular space toward the source of the chemoattractant.

One chemokine in particular that mediates inflammatory response is IL-8. IL-8 is a cytokine that promotes the recruitment and activation of neutrophil leukocytes and represents one of several endogenous mediators of the acute inflammatory response. In the past it was variously termed neutrophil-activating factor, monocyte-derived neutrophil chemotactic factor, IL-8, and neutrophil-activating peptide-1. The term "IL-8" has gained the widest acceptance and will be used herein.

Evidence of the involvement of IL-8 in inflammatory responses includes the observation that neutralizing antibodies to human IL-8 were shown to have a protective effect in inflammatory lung injury in rats.

Further, preliminary nonhuman primate studies have confirmed the activity of IL-8 on hematological parameters. IL-8 was administered by both bolus and continuous infusion to baboons. This resulted in a rapid, transient and severe granulocytopenia followed by granulocytosis that persisted as IL-8 levels remained detectable within the circulation. Histopathological examination revealed a mild to moderate neutrophil margination in the lung, liver and spleen which was of greater severity in animals receiving the continuous infusion of IL-8.

Also, high levels of intravascular IL-8 have been reported in systemic conditions such as septic shock.

Further, it is known that IL-8 binds with a higher affinity to CXCR-1 than to CXCR-2. On the other hand, a primary receptor for MCP-1 is CCR-2, which is expressed predominately on macrophages.

Another chemokine that mediates inflammatory response is MCP-1. Studies using animal macrophages have demonstrated the pivotal roles of MCP-1

in rheumatoid arthritis and atherosclerosis. Unfortunately, work on human macrophages has been hampered by the relative small number of these cells in human blood.

Historically, persons skilled in the pharmaceutical and medical arts have sought to increase levels of CSFs in patients, believing that CSFs provided therapeutic benefits to patients suffering from certain diseases and disorders. We have now unexpectedly discovered that CSFs synergistically enhance the chemoattractant effects of chemokines on recruitment of leukocytes to sites of inflammation. For example, it is shown below that G-CSF synergistically enhances the chemoattractant effects of IL-8 on the recruitment of neutrophils, and M-CSF synergistically enhances the chemoattractant effects of MCP-1 on the recruitment of monocytes. As IL-8 and MCP-1 are key mediators of inflammatory diseases, it would be desirable to identify substances capable of inhibiting the synergistic interactions of CSFs and chemokines for use in the treatment of diseases responsive to this inhibition.

We have now unexpectedly discovered useful methods for determining the ability of a compound, or a pharmaceutically acceptable salt thereof, to inhibit a synergistic interaction between a CSF and a chemokine, including a method for rapidly screening large numbers of such compounds. Accordingly, one embodiment of the present invention is a method for screening compounds, or pharmaceutically acceptable salts thereof, for inhibition of a synergistic interaction between a CSF and a chemokine. All that is needed to practice this embodiment of the present invention is to assay a potential inhibitor of said synergistic interaction according to the methods described below.

Another embodiment of the present invention is an inhibitor of a synergistic interaction between a CSF and a chemokine, which inhibitor is identified using a screening method of the present invention.

Further, another embodiment of the present invention is an inhibitor of a synergistic interaction between a CSF and a chemokine.

Still further, another embodiment of the present invention is a method of treating diseases and disorders responsive to inhibition of a synergistic interaction between a CSF and a chemokine.

-5-

Still further, another embodiment of the present invention is a pharmaceutical composition comprising an inhibitor of a synergistic interaction between a CSF and a chemokine, or a pharmaceutically acceptable salt thereof, and a pharmaceutically acceptable carrier, diluent, or excipient.

5 All that is needed to practice the present invention is to administer from one to six times daily a therapeutically effective amount of an inhibitor, or a pharmaceutically acceptable salt thereof, of a synergistic interaction between a CSF and a chemokine to a patient in need thereof for the treatment of inflammatory disorders and diseases responsive to inhibition of a synergistic
10 interaction between a CSF and a chemokine. Determination of proper dosage, pharmaceutical composition, and form of administration of the inhibitor is well within ordinary skill in the pharmaceutical and medical arts.

United States patent number 4,504,586 discloses murine-derived hybridoma tumor cell lines and monoclonal anti-Colony Stimulating Factor
15 Subclass Number 1 antibody substances produced by these cell lines. Use of said monoclonal antibody substances, alone or in combination, in immunological procedures for isolation of natural Colony Stimulating Factor Subclass Number 1 and for quantitative detection of colony Stimulating Factor Subclass Number 1 in fluid samples.

20 SUMMARY OF THE INVENTION

The present invention is connected to the discovery that CSFs appear to be critical for leukocyte recruitment, specifically polymorpho-nuclear neutrophil (PMN) and monocyte recruitment, and exhibit synergizing activity with chemokines. Inhibition of the synergistic interactions between CSFs and
25 chemokines useful in the present invention includes inhibition of the interaction between a CSF and a chemokine, and between a CSF and its receptor. Accordingly, inhibitors especially useful in the present invention include compounds capable of binding to a CSF, and thereby inhibiting the interaction between the CSF and a chemokine or the CSF receptor. Also especially useful
30 inhibitors in the present invention include compounds capable of binding to a CSF

receptor, and thereby inhibiting the interaction between the receptor at its CSF. Thus, inhibitors of the present invention include compounds which are antibodies directed against a CSF or a CSF receptor, small molecules capable of binding a CSF, and antagonists of CSF receptor.

5 One embodiment of the present invention is an inhibitor of a CSF which inhibits the synergistic effect of said CSF on chemokine-mediated inflammation, osteoporosis, an autoimmune disease, or atherosclerosis, comprising an agent which binds to a CSF, an agent which inhibits expression of a CSF, an antagonist of a colony stimulating factor receptor (CSFR), an antibody directed to a CSF or a
10 CSFR, or an agent which inhibits activation of a CSFR, or a pharmaceutically acceptable salt thereof.

A preferred embodiment of the present invention is an inhibitor of a CSF which inhibits the synergistic effect of said CSF on chemokine-mediated inflammation, osteoporosis, an autoimmune disease, or atherosclerosis,
15 comprising an agent which binds to a CSF, an agent which inhibits expression of a CSF, an antagonist of a CSFR, an antibody directed to a CSF or a CSFR, or an agent which inhibits activation of a CSFR, or a pharmaceutically acceptable salt thereof, wherein the CSF is a monocyte-colony stimulating factor (M-CSF).

Another preferred embodiment of the present invention is an inhibitor of a
20 CSF which inhibits the synergistic effect of said CSF on chemokine-mediated inflammation, osteoporosis, an autoimmune disease, or atherosclerosis, comprising an agent which binds to a CSF, an agent which inhibits expression of a CSF, an antagonist of a CSFR, an antibody directed to a CSF or a CSFR, or an agent which inhibits activation of a CSFR, or a pharmaceutically acceptable salt
25 thereof, wherein the chemokine is a beta-chemokine.

A more preferred embodiment of the present invention is an inhibitor of a CSF which inhibits the synergistic effect of said CSF on chemokine-mediated inflammation, osteoporosis, an autoimmune disease, or atherosclerosis,
comprising an agent which binds to a CSF, an agent which inhibits expression of a
30 CSF, an antagonist of a CSFR, an antibody directed to a CSF or a CSFR, or an agent which inhibits activation of a CSFR, or a pharmaceutically acceptable salt thereof, wherein the CSF is an M-CSF, the chemokine is monocyte chemotactic

protein-1 (MCP-1), and the inhibitor is an antibody directed to an M-CSF or an antibody directed to a monocyte-colony stimulating factor receptor (M-CSFR).

Especially preferred are said inhibitors which are monoclonal antibodies to an M-CSFR or M-CSF.

5 Another more preferred embodiment of the present invention is an inhibitor of a CSF which inhibits the synergistic effect of said CSF on chemokine-mediated inflammation, osteoporosis, an autoimmune disease, or atherosclerosis, comprising an agent which binds to a CSF, an agent which inhibits expression of a CSF, an antagonist of a CSFR, an antibody directed to a CSF or a CSFR, or an
10 agent which inhibits activation of a CSFR, or a pharmaceutically acceptable salt thereof, wherein the CSF is an M-CSF, the chemokine is MCP-1, and the inhibitor is an antagonist of an M-CSFR.

Another preferred embodiment of the present invention is an inhibitor of a CSF which inhibits the synergistic effect of said CSF on chemokine-mediated
15 inflammation, osteoporosis, an autoimmune disease, or atherosclerosis, comprising an agent which binds to a CSF, an agent which inhibits expression of a CSF, an antagonist of a CSFR, an antibody directed to a CSF or a CSFR, or an agent which inhibits activation of a CSFR, or a pharmaceutically acceptable salt thereof, wherein the CSF is a granulocyte-colony stimulating factor (G-CSF).

20 Another preferred embodiment of the present invention is an inhibitor of a CSF which inhibits the synergistic effect of said CSF on chemokine-mediated inflammation, osteoporosis, an autoimmune disease, or atherosclerosis, comprising an agent which binds to a CSF, an agent which inhibits expression of a CSF, an antagonist of a CSFR, an antibody directed to a CSF or a CSFR, or an
25 agent which inhibits activation of a CSFR, or a pharmaceutically acceptable salt thereof, wherein the chemokine is an alpha-chemokine.

A more preferred embodiment of the present invention is an inhibitor of a CSF which inhibits the synergistic effect of said CSF on chemokine-mediated
inflammation, osteoporosis, an autoimmune disease, or atherosclerosis,
30 comprising an agent which binds to a CSF, an agent which inhibits expression of a CSF, an antagonist of a CSFR, an antibody directed to a CSF or a CSFR, or an agent which inhibits activation of a CSFR, or a pharmaceutically acceptable salt thereof, wherein the CSF is a G-CSF, the chemokine is IL-8, and the inhibitor is

an antibody directed to a G-CSF or an antibody directed to a granulocyte-colony stimulating factor receptor (G-CSFR).

Especially preferred are said inhibitors which are monoclonal antibodies to a G-CSF or G-CSFR.

5 Another more preferred embodiment of the present invention is an inhibitor of a CSF which inhibits the synergistic effect of said CSF on chemokine-mediated inflammation, osteoporosis, an autoimmune disease, or atherosclerosis, comprising an agent which binds to a CSF, an agent which inhibits expression of a CSF, an antagonist of a CSFR, an antibody directed to a CSF or a CSFR, or an
10 agent which inhibits activation of a CSFR, or a pharmaceutically acceptable salt thereof, wherein the CSF is a G-CSF, the chemokine is IL-8, and the inhibitor is an antagonist of a G-CSFR.

Another preferred embodiment of the present invention is an inhibitor of a CSF which inhibits the synergistic effect of said CSF on chemokine-mediated
15 inflammation, osteoporosis, an autoimmune disease, or atherosclerosis, comprising an agent which binds to a CSF, an agent which inhibits expression of a CSF, an antagonist of a CSFR, an antibody directed to a CSF or a CSFR, or an agent which inhibits activation of a CSFR, or a pharmaceutically acceptable salt thereof, wherein the CSF is a GM-CSF.

20 Another embodiment of the present invention is a pharmaceutical composition, comprising an inhibitor of a CSF which inhibits the synergistic effect of said CSF on chemokine-mediated inflammation, osteoporosis, an autoimmune disease, or atherosclerosis, or a pharmaceutically acceptable salt thereof, and a pharmaceutically acceptable carrier, diluent, or excipient.

25 Another embodiment of the present invention is a method of treating inflammation, osteoporosis, an autoimmune disease, or atherosclerosis, comprising administering to a mammal in need thereof a therapeutically effective amount of an inhibitor of a CSF which inhibits the synergistic effect of said CSF on chemokine-mediated inflammation, osteoporosis, an autoimmune disease, or
30 atherosclerosis, or a pharmaceutically acceptable salt thereof.

Preferred is a method of treating inflammation, osteoporosis, an autoimmune disease, or atherosclerosis, comprising administering to a mammal in need thereof a therapeutically effective amount of an inhibitor of a CSF which

inhibits the synergistic effect of said CSF on chemokine-mediated inflammation, osteoporosis, an autoimmune disease, or atherosclerosis, or a pharmaceutically acceptable salt thereof, wherein the disease being treated is atherosclerosis.

Also preferred is a method of treating inflammation, osteoporosis, an autoimmune disease, or atherosclerosis, comprising administering to a mammal in need thereof a therapeutically effective amount of an inhibitor of a CSF which inhibits the synergistic effect of said CSF on chemokine-mediated inflammation, osteoporosis, an autoimmune disease, or atherosclerosis, or a pharmaceutically acceptable salt thereof, wherein the disease being treated is sepsis.

Also preferred is a method of treating inflammation, osteoporosis, an autoimmune disease, or atherosclerosis, comprising administering to a mammal in need thereof a therapeutically effective amount of an inhibitor of a CSF which inhibits the synergistic effect of said CSF on chemokine-mediated inflammation, osteoporosis, an autoimmune disease, or atherosclerosis, or a pharmaceutically acceptable salt thereof, wherein the disease being treated is asthma.

Also preferred is a method of treating inflammation, osteoporosis, an autoimmune disease, or atherosclerosis, comprising administering to a mammal in need thereof a therapeutically effective amount of an inhibitor of a CSF which inhibits the synergistic effect of said CSF on chemokine-mediated inflammation, osteoporosis, an autoimmune disease, or atherosclerosis, or a pharmaceutically acceptable salt thereof, wherein the disease being treated is an autoimmune disease.

Also preferred is a method of treating inflammation, osteoporosis, an autoimmune disease, or atherosclerosis, comprising administering to a mammal in need thereof a therapeutically effective amount of an inhibitor of a CSF which inhibits the synergistic effect of said CSF on chemokine-mediated inflammation, osteoporosis, an autoimmune disease, or atherosclerosis, or a pharmaceutically acceptable salt thereof, wherein the disease being treated is osteoporosis.

Also preferred is a method of treating inflammation, osteoporosis, an autoimmune disease, or atherosclerosis, comprising administering to a mammal in need thereof a therapeutically effective amount of an inhibitor of a CSF which inhibits the synergistic effect of said CSF on chemokine-mediated inflammation,

-10-

osteoporosis, an autoimmune disease, or atherosclerosis, or a pharmaceutically acceptable salt thereof, wherein the disease being treated is rheumatoid arthritis.

Also preferred is a method of treating inflammation, osteoporosis, an autoimmune disease, or atherosclerosis, comprising administering to a mammal
5 in need thereof a therapeutically effective amount of an inhibitor of a CSF which inhibits the synergistic effect of said CSF on chemokine-mediated inflammation, osteoporosis, an autoimmune disease, or atherosclerosis, or a pharmaceutically acceptable salt thereof, wherein the disease being treated is osteoarthritis.

The preferred use of the inhibitors of the present invention is for, but not
10 limited to, the treatment of atherosclerosis, osteoporosis, and chronic and acute inflammatory and autoimmune diseases such as SLE, GVHD, RA, IBD, asthma, and psoriasis.

Another embodiment of the present invention is a method for screening for an inhibitor of an M-CSF which inhibits the synergistic effect of said CSF on
15 chemokine-mediated inflammation, osteoporosis, an autoimmune disease, or atherosclerosis, comprising analyzing an (M-CSF)-stimulated monocyte population using a Fluorescent Activated Cell Sorter (FACS).

Preferred is a method for screening for an inhibitor of an M-CSF which inhibits the synergistic effect of said CSF on chemokine-mediated inflammation,
20 osteoporosis, an autoimmune disease, or atherosclerosis, comprising analyzing an (M-CSF)-stimulated monocyte population using a FACS, wherein the (M-CSF)-stimulated monocyte population is analyzed in whole blood after red blood cell lysis.

Also preferred is a method for screening for an inhibitor of an M-CSF
25 which inhibits the synergistic effect of said CSF on chemokine-mediated inflammation, osteoporosis, an autoimmune disease, or atherosclerosis, comprising analyzing an (M-CSF)-stimulated monocyte population using a FACS, wherein the screening method is a high throughput screening method.

Also preferred is a method for screening for an inhibitor of an M-CSF
30 which inhibits the synergistic effect of said CSF on chemokine-mediated inflammation, osteoporosis, an autoimmune disease, or atherosclerosis, comprising analyzing an (M-CSF)-stimulated monocyte population using a FACS,

wherein the (M-CSF)-stimulated monocyte population has also been stimulated by MCP-1.

Another preferred is a method for screening for an inhibitor of an M-CSF which inhibits the synergistic effect of said CSF on chemokine-mediated inflammation, osteoporosis, an autoimmune disease, or atherosclerosis, comprising analyzing an (M-CSF)-stimulated monocyte population using a FACS, wherein the (M-CSF)-stimulated monocyte population which has also been stimulated by MCP-1, is analyzed in whole blood after red blood cell lysis.

Another embodiment of the present invention is a method for screening for an inhibitor of a G-CSF which inhibits the synergistic effect of said CSF on chemokine-mediated inflammation, osteoporosis, an autoimmune disease, or atherosclerosis, comprising measuring binding of an (125) G-CSF to a G-CSFR in a (G-CSF)-stimulated neutrophil population.

Preferred is a method for screening for an inhibitor of a G-CSF which inhibits the synergistic effect of said CSF on chemokine-mediated inflammation, osteoporosis, an autoimmune disease, or atherosclerosis, comprising measuring binding of an (125) G-CSF to a G-CSFR in a (G-CSF)-stimulated neutrophil population, wherein the screening method is a high throughput screening method.

Another embodiment of the present invention is a method for screening for an inhibitor of a GM-CSF which inhibits the synergistic effect of said CSF on chemokine-mediated inflammation, osteoporosis, an autoimmune disease, or atherosclerosis, comprising measuring binding of an (125) GM-CSF to a GM-CSFR in a (GM-CSF)-stimulated neutrophil population or analyzing a (GM-CSF)-stimulated monocyte population using a FACS.

Another embodiment of the present invention is a method for screening for an inhibitor of a CSF which inhibits the synergistic effect of said CSF on chemokine-mediated inflammation, osteoporosis, an autoimmune disease, or atherosclerosis, the method comprising:

Step (a) Obtaining CSFR cDNA and corresponding (125)-CSF;

Step (b) Cloning the CSFR cDNA of Step (a) into a vector;

Step (c) Stably transfecting the vector of Step (b) into a hematopoietic cell line that resembles circulating leukocytes;

-12-

Step (d) Quantitating the transfected vector of Step (c) and measuring the binding of said (I¹²⁵)-CSF; and

Step (e) Screening agents for inhibition of CSF activity using a binding assay comprising the transfected vector of Step (c) and said (I¹²⁵)-CSF.

5 Another embodiment of the present invention is an inhibitor of a CSF that inhibits the synergistic effect of said CSF on chemokine-mediated inflammation, osteoporosis, an autoimmune disease, or atherosclerosis, which inhibitor is identified using one of the methods for screening for said inhibitors described above.

10 Because leukocytes are important mediators of inflammatory and immunoregulatory disorders and diseases, agents which inhibit or prevent leukocyte accumulation or activation by inhibiting the synergistic effect of a CSF on chemokine induced leukocyte-mediated inflammation, osteoporosis, an autoimmune disease, or atherosclerosis will be useful in such disorders and
15 diseases. The present invention, therefore, also provides an antagonist of CSF capable of inhibiting or minimizing the attractive capabilities of chemokines for leukocytes and of inhibiting or minimizing leukocyte accumulation and/or activation in vitro and in vivo.

20 The present invention further provides screens or assays for identifying agents that inhibits or otherwise hinders the binding of a CSF to a CSF receptor, for example, any agent that binds to a CSF or to a CSF receptor. Screens that can be employed in the identification of such antagonists/agonists are known to those of skill in the art. The different methods one could use to identify antagonists or agonists of CSF receptors include, but are not limited to: (1) Look for G-CSF
25 binding to its receptor on the cells over expressing the G-CSF receptor; (2) Look for down stream kinase activations and develop a high throughput assay; (3) Look for the transcription factor activations (such as STATs) as a functional read out in reporter gene assays amenable for high throughput screening.

30 The present invention also provides an agent that inhibits or otherwise hinders the production, release or action of a CSF, especially an agent as described above, for use as a medicament. The invention also provides the use of an agent that inhibits or otherwise hinders the production, release or action of a

-13-

CSF, especially an agent as described above, in the manufacture of a medicament for the treatment of asthma or another disease having an inflammatory component, particularly with accumulation of neutrophils, for example in ischemia reperfusion or acute respiratory distress or eosinophils, for example, rhinitis or eczema, especially allergic eczema syndrome.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1 to 21 of the accompanying drawings illustrate the present invention. The following is a brief description of the Figures. A more detailed description of the Figures is given below and in the Examples section of this specification.

Figure 1 illustrates that G-CSF synergizes IL-8 induced PMN chemotaxis.

Figure 2 illustrates that GM-CSF synergizes IL-8 induced PMN chemokines.

Figure 3 illustrates the dose response curve for IL-8 with fixed concentration of G-CSF.

Figure 4 illustrates that G-CSF does not synergize f-MLP induced neutrophil chemotaxis.

Figure 5 illustrates that G-CSF enhances IL-8 induced in vivo neutrophil intradermal recruitment.

Figure 6 illustrates the binding of 125 I G-CSF on polymorphonucleocytes (PMN).

Figure 7 illustrates that G-CSF neutralizing antibody inhibits G-CSF synergized chemotaxis.

Figure 8 illustrates that G-CSF pre-incubation alters PMN chemotactic response to IL-8.

Figure 9 illustrates that G-CSF does not alter IL-8 induced calcium flux.

Figure 10 illustrates that G-CSF does not increase IL-8 binding on PMNs.

Figure 11 illustrates that G-CSF pre-incubation does not alter IL-8 binding.

-14-

Figure 12 illustrates that G-CSF pre-incubation alters PMN response to IL-8.

Figure 13 illustrates that G-CSF potentiates both chemotactic and chemokinetic responses to IL-8.

5 Figure 14 illustrates that the three separate subpopulations of leukocytes can be detected by FACS.

Figure 15 illustrates that stimulation of human whole blood by MCP-1 causes an increase in the number of monocytes.

10 Figure 16 illustrates the time course of forward scatter detection of monocytes in response to stimulation of human whole blood by MCP-1.

Figure 17 illustrates a dose response curve plotting forward scatter detection of monocytes in human whole blood versus concentration of MCP-1.

Figure 18 illustrates the inhibitory effects of an anti-CCR-2 antibody on MCP-1 stimulation of human whole blood.

15 Figure 19 illustrates the effect of M-CSF on human monocyte size.

Figure 20 illustrates the lack of effect of M-CSF on human neutrophil size.

Figure 21 illustrates the synergistic effect of M-CSF and MCP-1 on human monocyte size.

DETAILED DESCRIPTION OF THE INVENTION

20 The present invention is directed to inhibitors of haematopoietic factors called colony stimulating factors and methods of treating diseases responsive to inhibition of colony stimulating factors (CSFs). The present invention is also directed to assays for screening inhibitors of CSF.

25 The present invention relates to the ability of CSFs to synergize the attractive capabilities of chemokines to leukocytes, preferably PMNs or monocytes. As discussed in the examples below, G-CSF appears to be critical for neutrophil recruitment and exhibits synergizing activity with IL-8, and M-CSF appears to be critical for macrophage recruitment and exhibits synergizing activity with MCP-1.

-15-

The inhibitors of the present invention have an important role in asthma and in other diseases having an inflammatory component where leukocyte accumulation and/or activation is a prominent feature, for example, an autoimmune disease, rheumatoid arthritis, osteoarthritis, atherosclerosis, rhinitis, and eczema, especially allergic eczema. Accordingly, agents that inhibit or otherwise hinder the production, release or action of CSFs have potential as selective therapeutic agents. Such agents and their therapeutic use are part of the present invention.

Preferred inhibitors of the present invention are inhibitors of M-CSF, including antibodies directed to M-CSF, especially monoclonal antibodies, and antagonists of M-CSFR. Preferred methods of treating of the present invention are methods employing the inhibitors of M-CSF of the present invention.

In order to provide an understanding of several of the terms used in the specification and claims, the following definitions are provided:

CSFs are defined to include the classic structurally distinguishable chemokines based on the C-terminal cysteine arrangement, as opposed to the peptide f-Met-Leu-Phe (f-MLP), which does not belong to the classic chemokine classes.

The term "comprising", which is synonymous with the terms "including", "containing", or "characterized by" is inclusive or open-ended and does not exclude additional, unrecited elements or method steps from the scope of the invention that follows.

The phrase "consisting of" is closed-ended and excludes any element, step, or ingredient not specified in the description of the invention that follows.

The phrase "consisting essentially of" limits the scope of the invention that follows to the specified elements or steps and those further elements or steps that do not materially affect the basic and novel characteristics of the invention.

EC₅₀: The effective concentration of an agent required to produce 50% of a maximal response.

Biological Activity: The term biological activity is a function or set of functions performed by a molecule in a biological context (i.e., in an organism or an in vitro surrogate or facsimile model). For IL-8 or other alpha chemokine

-16-

biological activity is characterized by its chemotactic activity (preferably PMNs, but may also include T lymphocytes and/or monocytes/macrophages).

Leukocyte: A white blood cell which may be a granulocyte, lymphocyte, or monocyte.

5 Chemokine: A biological molecule capable of attracting a subset of cell population from the circulating blood to the site of its presence in a gradient dependent fashion. There are two classes of chemokines, the members of each class share an organizing primary sequence motif. The alpha chemokines, such as IL-8, neutrophil-activating protein-2 (NAP-2), and melanoma growth stimulatory
10 activity protein (MGSA) are chemotactic primarily for neutrophils, whereas beta chemokines, such as RANTES (regulation-upon-activation, normal T expressed and secreted), MIP-1 alpha (macrophage inflammatory protein), MIP-1 beta, MCP-1 (monocyte chemotactic protein-1), MCP-2, and MCP-3 are chemotactic for monocytes, T-cells, eosinophils, and basophils.

15 PMN: Polymorpho-nuclear neutrophils represent the mature form of circulating leukocyte population that has evolved from the granulocytic lineage and that goes through the developmental stages of myeloblast, promyelocyte, myelocyte and metamyelocyte. PMNs primarily refer to neutrophils. Other cell types include the monocyte, which also comes from the same granulocyte lineage
20 and evolves from promonocyte to monocyte.

Assay or Screen: A method used to evaluate the efficacy (agonism or antagonism) of the chemical compounds or biological factors in a given assay system. The system may be amenable for high throughput efficiency.

25 Modulate: An increase or decrease seen in a set pattern of activity in a system upon addition or deletion of another factor in the same system.

The phrase "autoimmune disease" means the diseases classified as "Highly probably" or Probable" in TABLE 20-3. PUTATIVE AUTOIMMUNE DISORDERS of The Merck Manual of Diagnosis and Therapy, 16th edition, Robert Berkow, ed., Merck Research Laboratories, Rahway, New Jersey, 1992,
30 page 340, which is hereby incorporated herein by reference. Diseases classified as highly probable include, to name a few, systemic lupus erythematosus, Grave's disease, myasthenia gravis, insulin resistance, and autoimmune hemolytic anemia. Diseases classified as probable include, to name a few, rheumatoid arthritis,

scleroderma with anti-collagen antibodies (Abs), pernicious anemia, and some cases of diabetes mellitus.

The term "atherosclerosis" means diseases in which the walls of medium- and large-diametered arteries become thickened and lose elasticity (see the Merck
5 Manual of Diagnosis and Therapy, supra., 1992; 409-413).

Inflammatory diseases and disorders are diseases and disorders with an inflammatory component. Preferred inflammatory diseases and disorders include atherosclerosis, rheumatoid arthritis, osteoarthritis, asthma, and autoimmune diseases.

10 Osteoporosis is a generalized, progressive diminution in bone tissue mass per unit volume, causing skeletal weakness. Bone resorption is increased in osteoporosis, while rate of bone formation appears to be normal, although it may be defective in nature. Risk factors for osteoporosis include rheumatoid arthritis and chronic obstructive pulmonary disease (COPD), both of which are conditions
15 with an inflammatory component.

It is to be appreciated that as used herein the phrases "antibodies to", "antibodies directed against", and "antibodies capable of binding with" are used interchangeably.

Further, it is to be appreciated that the terms "antibody" and "antibodies,"
20 as used herein, mean a human antibody or antibodies, respectively, which is/are directed against a human CSF. Development of such antibodies may be carried out, for example, by employing a mouse-derived cell line wherein mouse antibody genes have been knocked out and human antibody genes inserted instead. Mouse and other nonhuman, nonprimate-derived antibodies to a human CSF are not part
25 of the present invention as these antibodies, when administered to a human, will cross react, usually in about 20 to 25 days. This cross reaction means the human host develops an immunological reaction to the nonhuman, nonprimate derived antibodies, which prohibits use of said antibodies in the treatment of human disease.

30 Inhibitors that affect the interaction of a CSF with CSF receptors, for example, by binding to a CSF or to a CSF receptor, inhibit a chemokine's attractive abilities to leukocytes and are part of the present invention. An example of such an inhibitor is receptors themselves which, on administration, can bind a

-18-

CSF and prevent its interaction with naturally-occurring receptors. Such inhibitory receptors may be soluble or insoluble. Receptors which are not involved in cell activation may be bound to, or induced on, cells. Such receptors may also be used to remove endogenous CSF.

5 Further examples of inhibitors that inhibit the interaction of CSF with CSF receptors are receptor antagonists and antibodies, both antibodies directed against (capable of binding with) a CSF and antibodies directed against a CSF receptor. Especially preferred antibodies are monoclonal antibodies. Any other inhibitor that inhibits or otherwise hinders the binding of a CSF to a CSF receptor,
10 including, for example, any other inhibitor that binds to a CSF or to a CSF receptor, also has therapeutic potential. Further, inhibitors that have therapeutic potential are also those that prevent or inhibit activation of CSF receptors.

Further, inhibitors that inhibit or otherwise hinder the action of CSFs are those that change the structure of a CSF such that it is no longer able to bind to a CSF receptor. One example of such an inhibitor is an enzyme or other agent that
15 degrades CSF specifically.

Receptor promiscuity is common among CSFs, so although it is essential that a receptor is capable of binding a CSF, the receptor need not necessarily be M-CSF-specific or G-CSF-specific. For example, a receptor may bind GM-CSF,
20 M-CSF, G-CSF, and/or other leukocyte attractant CSF.

As indicated above, possibilities for therapeutic intervention include the use of a receptor to which a CSF binds, especially a soluble receptor. It may be advantageous to use a CSF-specific receptor. Further possibilities for therapeutic intervention include receptor antagonists, for example, based on 3-dimensional
25 structures or the amino acid sequences of CSFs and/or of CSF receptors, and agents found to inhibit CSF or other agonists binding to or activating CSF receptors.

Inhibitors that prevent or inhibit CSF synthesis or release may also be used therapeutically. Such agents and their use are also part of the present invention.

30 All inhibitors of CSF activity, synthesis, and release, including soluble receptors, antibodies, antagonists and inhibitors of agonist binding, and their use are part of the present invention.

It is also to be appreciated that the inhibitors of the present invention may have chiral centers, in which case all stereoisomers thereof, both separately and as racemic and/or diastereoisomeric mixtures, are included.

Some of the inhibitors of the present invention are capable of further forming nontoxic pharmaceutically acceptable acid addition and/or base salts. All of these forms are within the scope of the present invention.

For example, pharmaceutically acceptable acid addition salts of the inhibitors of the present invention include salts derived from inorganic acids such as hydrochloric, nitric, phosphoric, sulfuric, hydrobromic, hydriodic, hydrofluoric, phosphorous, and the like, as well as the salts derived from organic acids, such as aliphatic mono- and dicarboxylic acids, phenyl-substituted alkanolic acids, hydroxy alkanolic acids, alkanedioic acids, aromatic acids, aliphatic and aromatic sulfonic acids, etc. Such salts thus include sulfate, pyrosulfate, bisulfate, sulfite, bisulfite, nitrate, phosphate, monohydrogenphosphate, dihydrogenphosphate, metaphosphate, pyrophosphate, chloride, bromide, iodide, acetate, trifluoroacetate, propionate, caprylate, isobutyrate, oxalate, malonate, succinates, suberate, sebacate, fumarate, maleate, mandelate, benzoate, chlorobenzoate, methylbenzoate, dinitrobenzoate, phthalate, benzenesulfonate, toluenesulfonate, phenylacetate, citrate, lactate, maleate, tartrate, methanesulfonate, and the like. Also contemplated are salts of amino acids such as arginate and the like and gluconate, galacturonate (see, for example, Berge S.M., et al., "Pharmaceutical Salts," *Journal of Pharmaceutical Science*, 1977;66:1-19).

The acid addition salts of basic inhibitors of the present invention are prepared by contacting the free base form of the inhibitors with a sufficient amount of the desired acid to produce the salt in the conventional manner. Usually, 1 mol equivalent of desired acid is contacted with 1 mol equivalent of the inhibitor.

Pharmaceutically acceptable base salts of inhibitors of the present invention are formed with metal cations such as alkali and alkaline earth metal cations or organic ammonium compounds. Examples of metal cations used are cations of sodium, potassium, magnesium, calcium, and the like. An organic ammonium compound is formed by protonation of the corresponding organic amine. Examples of suitable organic amines are N,N-dibenzylethylenediamine,

-20-

chloroprocaine, choline, diethanolamine, dicyclohexylamine, ethylenediamine, N-methylglucamine, and procaine (see, for example, Berge, supra., 1977).

Base salts of acidic inhibitors of the present invention are prepared by contacting the free acid form of the inhibitor with a sufficient amount of a suitable base that provides the desired metal cation or organic ammonium compound to produce a salt in the conventional manner. A suitable base that provides a desired metal cation includes alkali and alkaline metal cation hydroxides and carbonates. A suitable base that provides a desired organic ammonium compound is the free base of the corresponding organic amine. Usually, 1 mol equivalent of a suitable base that provides the desired metal cation or organic ammonium compound is contacted with 1 mol equivalent of the inhibitor.

Certain of the compounds of the present invention can exist in unsolvated forms as well as solvated forms, including hydrated forms. In general, the solvated forms, including hydrated forms, are equivalent to unsolvated forms and are intended to be encompassed within the scope of the present invention.

Inhibitors of the present invention may be identified using in vivo and in vitro assays based on inhibition of chemoattraction and/or accumulation and/or activation of leukocytes by CSFs. Some general methods for testing the activity of a compound for an inhibitory effect on the activity of a chemoattractant chemokine in vitro are known. Such assays may be used to determine the inhibitory action of a putative inhibitor on in vitro effects induced in leukocytes by the synergistic activity of CSFs on chemokines.

Examples of in vitro and in vivo assays both for the determination of CSF activity and for the determination of CSF inhibitory activity are described herein. For example, Example 1 gives a detailed protocol for an in vitro assay of the present invention. The assays described herein may be used as such, or may be modified as required. Assays may be used alone or in combination with other assays known to those skilled in the art to establish CSF and CSF-inhibitory activity. A putative inhibitor of the present invention may be any of the types of molecules described above, including receptors, for example, soluble receptors, antibodies, and antagonists and inhibitors of agonist binding. A protocol screening assay for G-CSF receptor antagonists is described in Example 2.

-21-

A protocol screening assay for inhibitors of the synergistic effect of M-CSF on a chemokine-induced, monocyte-mediated inflammation, osteoporosis, an autoimmune disease, or atherosclerosis is described in Example 3.

Assays for inhibitors of a CSF include immunoassays, particularly
5 enzyme-linked immunosorbent assays (ELISAs). The invention provides, for example, an immunoassay for an antigen, characterized in that the antigen is a CSF, and also provides an immunoassay for an antibody, characterized in that the antibody is an anti-CSF antibody. The invention also provides assays for CSFs that are analogous to immunoassays for CSFs, but that use a specific-binding
10 partner other than an antibody. In such specific-binding partner assays, a CSF receptor, including a soluble CSF receptor, may be used instead of an anti-CSF antibody.

In an immunoassay, an anti-CSF antibody may, for example, be coated on a solid surface to enable capture and hence detection of CSF. An anti-CSF
15 antibody may be used in an assay for the detection of antibodies to CSF, for example, in a competitive antibody assay. A labeled CSF or a derivative thereof, for example, a recombinant CSF or a synthetic peptide comprising part of the amino acid sequence of an CSF may be used in a competitive antigen assay for CSF or may be used to coat a solid surface in a capture assay for antibodies to
20 CSF. The many different types of assay format are well described in the literature of the art, see for example "ELISA and Other Solid Phase Immunoassays, Theoretical and Practical Aspects" eds. Kemeny D.M. and Challacombe S.J., John Wiley, 1988:(36). Assays using an CSF receptor instead of an anti-CSF antibody may be carried out analogously.

25 The following examples are provided to further illustrate the present invention and are not intended to limit the invention beyond the limitations set forth in the appended claims.

EXAMPLE 1

Materials and Methods

30 Reagents & Animals: For all experiments, male New Zealand White rabbits (3.0-3.5 Kg) purchased from Charles River Breeding Lab (Wilmington, MA) were used. The animals were fed standard lab rabbit CHOW (Ralston Purina

-22-

Company, St. Louis, MO) and housed according to institutional guidelines. G-CSF was obtained from R&D Laboratories (Minneapolis, MN); transwell plates for chemotaxis were obtained from Corning-Costar (Corning, NY); LYMPHOPREP (Nyegaard & Co. A.S., Oslo, Norway) was obtained from Nycomed Pharma A.S., Oslo, Norway; prostaglandin E₂ (PGE₂) was obtained from Caymen Chemical (Ann Arbor, MI); bradykinin was obtained from Sigma Chemical Co. (St. Louis, MO); and ¹²⁵I-IL-8 was obtained from Amersham Life Science, Buthinghamshire, England. IL-8 was a kind gift from Leukocyte, Inc. (Boston, MA).

Chemotaxis Assay: Peripheral circulating neutrophils were isolated from the blood of normal healthy volunteers. Heparinized venous blood was dextran sedimented followed by centrifugation over LYMPHOPREP and hypotonic lysis of contaminating red blood cells. Isolated neutrophil pellet was resuspended in supplemented RPMI media (sodium bicarbonate free RPMI with 50 mM of HEPES and 0.2% BSA). Chemotaxis assay was performed in the 3 μ M transwell chambers. The cells were placed in the top chamber at a density of 1×10^6 /well, and the chemoattractant (interleukin-8 \pm G-CSF) was placed in the bottom chamber. In the G-CSF preincubation studies, cells were treated with G-CSF in the top chamber for the indicated time periods and then were subsequently exposed to IL-8 in the bottom chamber. In studies to assess chemokinesis, IL-8 and/or G-CSF was placed instead in the top chamber. Migration of neutrophils into the bottom chamber in response to the chemoattractant was monitored. The migrated neutrophils were quantitated by FACScan (Beckton Dickinson Immunocytometry Systems, San Jose, CA) analysis.

In Vivo Recruitment Assay: At t = 0, rabbits received 14.5 ng/kg (10 μ Ci) ¹²⁵I- IL-8 IV. At t = 15 minutes rabbits were anesthetized with isoflurane and injected intradermally (100 μ L/site) with 0.01-3.3 μ g IL-8 or IL-8 and G-CSF (100 pM). The vehicle consisted of sterile saline supplemented with 0.2% bovine serum albumin and 0.01 mM PGE₂ and 0.1 μ M bradykinin. At t = 75 minutes, rabbits were euthanized with 1 mL IV dose of BEUTHANASIA (Burns Pharmaceuticals, Oakland, CA): -D Special (Shering-Plough Animal Health,

-23-

Kenilworth, NJ), and skin biopsies were obtained using a 6.35 mm diameter punch (O'Brien Consolidated Industries, Lewiston, ME). The biopsies were weighed, and the radioactivity was determined using a gamma counter (Packard Model 05005, Downers Grove, IL).

5 Calcium Flux Assay: Calcium flux in response to IL-8 with or without G-CSF was assessed using standard fluorescent emission protocol. Briefly, neutrophils were loaded with 4 μ M of Fluo-4, for one hour at 37°C. In a 96-well plate approximately 300,000 cells/well were preincubated with or without G-CSF (0.1 pM to 1000 pM) for the respective time periods indicated and then were
10 subsequently stimulated with IL-8. Intracellular calcium flux in the cell was measured using FLIPR³⁸⁴ (Molecular Devices, Sunnyvale, CA).

15 Binding Assay: The IL-8 and G-CSF binding assays were performed on the isolated human neutrophils using 0.3 mL micro sedimentation tubes (Sarstedt, Newton, NC). A small amount (9.5 μ L) of sucrose solution (0.4%) was placed through the narrow tip of the tube by centrifugation. The reaction mix was layered gently on the sucrose solution to allow an air bubble in between the two solutions. In IL-8 binding studies, a total of 40,000 cells with 0.5 nM of hot IL-8 were used in each reaction. In cold competition studies, excess of 500 fold cold IL-8 was added. To quantitate the G-CSF binding on PMN, the cell number was varied
20 from 50,000 to 1,000,000 per reaction with 5 nM of ¹²⁵I G-CSF. In both binding studies, the cells were incubated with hot ligand for 3 hours and were pelleted at 10,000 RPM for 2 minutes. The sucrose layer was used to separate the bound from the unbound radio-labeled ligand. The cell pellet was cut out with a razor blade and the radioactive counts in the cell pellet were measured using a gamma
25 counter.

30 In Vivo Experiment Data Analysis: In each experiment, triplicate samples were obtained for each experimental condition. Counts were adjusted by first subtracting out baseline control values, normalizing for differences in tissue weight, and adjusting the activity to that of a sample with a theoretical 100 mg tissue weight. In dose-response studies where the 1- μ g dose of IL-8 was used, the weight-normalized background (vehicle-treated skin) was subtracted from weight-normalized treated samples, and the data was then expressed as a percent

-24-

maximal response to the 1- μ g dose of IL-8. The dose of 1 μ g of IL-8 typically produces the approximate maximal recruitment response to IL-8. Normalizing the data with this method reduced the interanimal variability.

Statistical Analysis: All data are presented as mean \pm standard deviation.

5 Student t-test was used to compare between groups using JMP (SAS Institute Inc, Cary, NC) Version 3.0, statistical analysis software.

G-CSF was traditionally known to be a hematopoietic growth factor and also known to enhance functional capacities of the neutrophils through maturation of the cell. However, surprisingly, in G-CSF knockout mice, the circulating
10 mature neutrophil number was less, but maturation was not defective, attributing additional roles for the traditionally known hematopoietic growth factor. Similar observations were made in G-CSF receptor knockout mice. In addition, it was observed that the neutrophils from G-CSF receptor knockout mice do not chemotax in response to IL-8 or f-MLP. Based on these studies, we proposed that
15 a synergism exists between G-CSF and other chemokines for its functional activation, in vivo. Studies indicating that neutralizing G-CSF antibodies inhibit the G-CSF induced synergism supported the G-CSF-specific mechanism. Our studies, in vivo and in vitro support the synergistic mechanisms that exist between these molecules. In vivo, in inflammatory situations, picomolar concentrations of
20 G-CSF are found at the site along with nanomolar concentrations of chemokines. Hence, it is quite possible that the synergistic mechanisms that we found here in the study are biologically significant. Hence, blocking the synergism using strategies that antagonize G-CSF or the receptor to prevent inflammation would be a useful tool to prevent the inflammatory process.

25

EXAMPLE 2

Protocol for a potential screening assay for G-CSF receptor antagonists:

This assay utilizes the binding properties of G-CSF directly to its receptor on the cells.

1. Obtain G-CSF receptor cDNA from the human genomic DNA synthesized
30 from human neutrophils. The cDNA can be obtained by polymerase chain

-25-

reaction (PCR) using primers aligned to the 5' and 3' ends of the mRNA sequence that is readily available in the IMPATH GENE BANK CANCER TISSUE/PERIPHERAL BLOOD REPOSITORY (IMPATH, Inc, Franklin, MA).

- 5 2. Clone the G-CSFR cDNA into a plasmid such as pcDNA 3.1 and stably transfect into a hematopoietic cell line that closely resembles the circulating leukocytes. Stably integrated clones can be screened by using an antibiotic resistance marker such as gentamycin (G418).
- 10 3. Using radiolabeled G-CSF, the expression of this transfected G-CSFR can be quantitated and the binding of G-CSF can be measured. The binding is performed using radiolabeled (I^{125}) G-CSF. (I^{125})G-CSF is incubated with the stably transfected cells in a tube for 3 hours in the presence or absence of cold (i.e., not radiolabeled) G-CSF. After 3 hours, the unbound fraction of the radiolabeled G-CSF is separated using sucrose gradient separation technique. The cells with the bound radiolabeled G-CSF are pelleted, and the amount of incorporated radioactive material is measured using a scintillation counter. The amount of scintillation count is proportional to the amount of bound radiolabeled G-CSF, and a high ratio of radiolabeled G-CSF (to radiolabeled G-CSF plus non-radiolabeled G-CSF) binding indicates less nonspecific binding. Based on the binding, further experiments selecting for high expressing clones are performed.
- 15 4. To screen for G-CSFR antagonists, cells are first preincubated with the inhibitor of the present invention for 30 minutes, at which point, the radiolabeled G-CSF is added, and incubation is continued for 3 hours more. An antagonist will decrease the binding of the G-CSF to its receptor, and this decrease will be evident from the decreased binding of radiolabeled G-CSF on the cells.
- 20 5. The effective concentrations of the antagonists will be evaluated by performing a dose-response curve on the inhibitors of the present invention. An antagonist might represent a molecule that binds to the receptor and does not allow the receptor's ligand to bind, or it could be a
- 25
- 30

-26-

compound that binds to the receptor's ligand and does not let the ligand bind to the receptor.

Similar protocols can be developed to screen for G-CSF antagonists using kinase assay systems or gene transcriptional activation systems using reporter constructs. All of these protocols are amenable for high throughput screening.

Protocols for screening inhibitors of the synergistic effect of M-CSF on a chemokine-induced monocyte-mediated inflammation, osteoporosis, an autoimmune disease, or atherosclerosis are described below, including Example 3.

EXAMPLE 3

The assay of Example 3 provides a method for directly measuring activation of human monocytes in human whole blood using a Fluorescent Activated Cell Sorter (FACS) instrument. The FACS uses laser excitation to detect leukocytes by measuring the autofluorescence of leukocyte DNA. The assay of Example 3 provides a method of detecting the three main classes of leukocytes (i.e., granulocytes, lymphocytes, and monocytes) using forward scatter (FSC) to determine characteristic cell sizes and using side scatter (SCC) to determine characteristic cell granularities.

In response to stimulation by a chemokine and/or M-CSF, the monocyte cell population as measured by FSC (i.e., size) increases. The measured response is indicative of either a cell shape change (e.g., ruffling of cell membrane) and/or a homotypic aggregation of monocyte cells. Both cell shape change and homotypic aggregation of monocyte cells occur during initial activation, adhesion, and transendothelial migration of chemokine-activated monocytes.

In the assay, a volume (90 μ L) of human whole blood was combined with an aqueous solution (10 μ L) containing a known concentration of M-CSF (or optionally M-CSF and MCP-1 if direct measurement of the inhibition of the synergistic effect of M-CSF on MCP-1 induced chemotaxis is desired), and the mixture was incubated at 37°C for 30 minutes. A 0.5% solution of formalin (100 μ L) was added, and the cells were fixed for 5 minutes. Red blood cell lysis was then achieved by adding 1 mL of lysis buffer and incubating the mixture at

-27-

37°C for 5 minutes. An aliquot of the mixture was then withdrawn, and its monocyte population analyzed using FACS.

The method of the present invention which is the assay of Example 3 provides a number of advantages over traditional methods of studying monocytes. First, the assay is substantial easier to use than current methods as isolation of monocytes from whole blood is no longer necessary. Second, the assay provides a method for analyzing monocytes in their normal environment of serum, other blood cell types, serum esterase, and potential protein binding whereas certain literature methods assay monocytes in isolated peripheral blood mononuclear cells (PBMCs). Third, isolation of PBMCs ages and activates the monocytes whereas the present assay does not so activate the monocytes. Fourth, the assay of Example 3 allows monocytes to be directly used as a surrogate marker for an inflammatory or atherosclerotic condition in in vivo animal and human efficacy studies.

In a procedure analogous to that described above in Example 2, antagonists of an M-CSF receptor may be identified as described below in Example 4.

EXAMPLE 4

This assay utilizes the binding properties of M-CSF to a soluble M-CSF receptor. The assay may be employed to screen single compounds or used in high throughput screening mode to screen many compounds rapidly.

1. Obtain soluble M-CSF receptor from R&D Systems Inc., Minneapolis, Minnesota.

2. Biotinylate the soluble M-CSFR from Step 1.

3. Using radiolabeled M-CSF, the expression of this biotinylated, soluble M-CSFR can be quantitated and the binding of M-CSF can be measured.

The binding is performed using radiolabeled (I^{125}) M-CSF. Preincubate the biotinylated, soluble M-CSFR from Step 2 in a tube or multi-well plate for 30 minutes. Add (I^{125})-M-CSF, and incubate for 2 hours in the presence or absence of cold (i.e., not radiolabeled) M-CSF. Pass the mixture through streptavidin-coated scintillation beads to bind the

-28-

- biotinylated, soluble M-CSFR. The amount of incorporated radioactive material is measured using a scintillation counter. The amount of scintillation count is proportional to the amount of bound radiolabeled M-CSF, and a high ratio of radiolabeled M-CSF (to radiolabeled M-CSF plus non-radiolabeled M-CSF) binding indicates less nonspecific binding.
- 5
4. To screen for M-CSFR antagonists, preincubate the biotinylated, soluble M-CSFR from Step 2 with a compound or compounds of the present invention (or for that matter any compound(s) for which it is desired to measure inhibition of M-CSF) for 30 minutes, at which point, the
- 10 radiolabeled M-CSF is added, and incubation is continued for 2 hours more. An antagonist will decrease the binding of the M-CSF to its receptor, and this decrease will be evident from the decreased binding of radiolabeled M-CSF on the cells.
5. The effective concentrations of the antagonists will be evaluated by performing a dose-response curve on the inhibitors of the present invention. An antagonist might represent a molecule that binds to the
- 15 receptor and does not allow the receptor's ligand to bind, or it could be a compound that binds to the receptor's ligand and does not let the ligand bind to the receptor.

20 Similar protocols can be developed to screen for M-CSF antagonists using kinase assay systems or gene transcriptional activation systems using reporter constructs. All of these protocols are amenable for high throughput screening.

DETAILED DESCRIPTION OF THE DRAWINGS

As shown above, several experiments were conducted to confirm that

25 G-CSF synergizes the IL-8 induced chemotaxis in vivo and in vitro. The methods and materials employed to conduct the studies are discussed in detail above in Example 1, which serves to guide those skilled in the art in understanding the inventions described herein. The results are summarized in Figures 1 through 13. The synergy of G-CSF on IL-8 chemotaxis is demonstrated in Figure 1 where

30 increases in G-CSF concentration, on a picomolar level, increases the chemotactic

-29-

response of neutrophils. Similarly, the synergy of GM-CSF on IL-8 chemotaxis is demonstrated in Figure 2. Figure 3 illustrates the dose response curve for IL-8 with fixed G-CSF concentration and demonstrates a 10-fold increase in neutrophil response to a combination of IL-8 and G-CSF. Figure 4 shows that G-CSF does not synergize the f-MLP induced neutrophil chemotaxis, indicating an IL-8, and chemokines functionally and/or structurally similar to IL-8, specific phenomenon. Figure 5 shows that the synergy is not limited to in vitro studies, but is also shows that G-CSF enhances in vivo neutrophil intradermal recruitment. Figure 6 illustrates and confirms the binding of I^{125} G-CSF to neutrophils. Figure 7 illustrates that G-CSF neutralizing antibody inhibits G-CSF synergized chemotaxis. Figure 8 illustrates that G-CSF preincubation alters PMN chemotactic response to IL-8. Figures 9 and 10 demonstrate that G-CSF synergism is independent of its effects on IL-8 binding or IL-8 induced calcium flux, suggesting involvement of a signaling mechanism. The study was conducted by employing radiolabeled I^{125} G-CSF and studying its effects on polymorpho nuclear neutrophils (PMN). Figure 11 illustrates that G-CSF pre-incubation does not alter IL-8 binding. Figure 12 illustrates that G-CSF pre-incubation alters PMN response to IL-8. Figure 13 illustrates that the potentiation effects of G-CSF on IL-8 induced migration were approximately equal on both the chemotactic and chemokinetic responses. The enhancement of IL-8 induced migration was evident irrespective of whether G-CSF was placed in the top or bottom of the chemotaxis chamber.

In summary, the data illustrated in Figures 1 through 13 show that G-CSF at biologically relevant concentrations (10-1000 pM) significantly potentiates IL-8 specific chemotaxis, but G-CSF does not affect f-MLP induced chemotaxis. Further, the data show that these acute effects of G-CSF are not mediated via effects on IL-8 binding or IL-8-induced calcium flux. However, the data also show that preincubation of neutrophils, in vitro with G-CSF for 120 minutes desensitized the neutrophils for subsequent IL-8 activation.

Several experiments were conducted that demonstrate M-CSF synergized MCP-1 induced chemotaxis. The methods and materials employed to conduct the studies are described above in Example 3. The results are illustrated in Figures 14

-30-

through 21. In Figure 14, a FACS dot plot of FSC versus SSC on unstimulated human whole blood shows that the three main classes of leukocytes can be detected, with normal monocytes plotted in the quadrant labeled R1 and monocytes with greater FSC (i.e., size) plotted in the quadrant labeled R2. The effect of MCP-1 stimulation on monocyte size is shown in Figure 15, where stimulation by MCP-1 increases the number of monocytes with greater FSC (i.e., size). The effect on FSC as a percent of a control value over time in response to stimulation of monocytes by MCP-1 at a concentration of 50 nM is shown in Figure 16 to increase until a plateau is reached nearly 30 minutes after initial exposure. In Figure 17, a dose response curve illustrates that monocyte size, as measured by FSC as a percent of a control value, increases with increasing doses of MCP-1 until a peak is reached at about 100 nM of MCP-1. The EC₅₀ in this experiment was 2 nM of MCP-1. Figure 18 illustrates that an antibody to the chemokine receptor for MCP-1, i.e., an antibody to the CCR-2 receptor, inhibits MCP-1 stimulation of monocytes as shown by the dose response curves of monocyte size, as measured by FSC as a percent of a control value, versus concentration of MCP-1. As expected, the shift of the MCP-1 plus antibody curve is parallel to the MCP-1 alone curve, which demonstrates that the observed effect is MCP-1 dependent and not due to a contaminant such as an endotoxin. In Figure 19, a dose response curve illustrates that monocyte size, as measured by FSC as a percent of a control value, increases with increasing doses of M-CSF, with an EC₅₀ for M-CSF of 253 pM. M-CSF is shown in Figure 20 to have no effect on the shape of human neutrophils. Figure 21 illustrates the synergistic effect of M-CSF on MCP-1 induced change in human monocyte shape, as the number of "large" monocytes in the presence or absence of M-CSF versus change in MCP-1 concentration. At a concentration of 1 pM of M-CSF, no statistically significant change in the dose response curve of MCP-1 was observed. However, at concentrations of 10 pM and 100 pM of M-CSF, the dose response curves were shifted towards lower concentrations of MCP-1 (i.e., to the left) by about 1 to 2 log units (i.e., by a factor of 0.1 to 0.01), and the overall response of monocytes to MCP-1 was significantly increased.

-31-

In summary, the data illustrated in Figures 14 through 21 show MCP-1 directly acts on monocytes in human whole blood in a time- and concentration-dependent manner. M-CSF alone much more potently induces changes in the shape of monocytes as compared to its effect on changes in the shape of neutrophils. M-CSF synergistically increases the potency of MCP-1 induced effects on monocytes from an EC₅₀ of 2 nM to and EC₅₀ of 20 pM. In vivo, picomolar concentrations of M-CSF and nanomolar concentrations of MCP-1 are found. The synergistic effects described above for M-CSF and MCP-1 are biologically significant, and inhibitors of the synergistic effect of M-CSF on a chemokine such as, for example, MCP-1 involved in monocyte-mediated inflammation, osteoporosis, an autoimmune disease, or atherosclerosis would be valuable therapeutic agents for the treatment thereof.

The dose and dosage regiment of an inhibitor of the present invention that is suitable for administration to a particular patient can be determined by a physician considering the patient's age, sex, weight, general medical condition, and the specific condition and severity thereof for which the inhibitor of the present invention is being administered; the route of administration of the inhibitor; the pharmaceutical carrier with which the inhibitor may be combined; and the inhibitor's biological activity.

Generally, intravenous subcutaneous or transmuscular injection of 1-500 μ Mol of CSF antagonizing compounds/kg body weight, by bolus injection, by infusion over a period of about 5 minutes to about 60 minutes, or by continuous infusion is sufficient for therapeutic efficacy. Aerosol inhalation of 0.1 to 2 mg of an inhibitor of the present invention/kg body weight is also sufficient for efficacy.

Intravenous, subcutaneous or intramuscular administration, by bolus injection or continuous infusion, is preferred for use of some of the inhibitors of the present invention in treatment of autoimmune or inflammatory disease.

The inhibitors of the present invention, or a pharmaceutically acceptable salt thereof, can be combined, over a wide concentration range (e.g., 0.001 to 11.0 wt %) with any standard pharmaceutical carrier (e.g., physiological saline, THAM solution, or the like) to facilitate administration by any of various routes

-32-

including intravenous, subcutaneous, intramuscular, oral, or intranasal, including by inhalation.

The inhibitors of the present invention can be prepared and administered in a wide variety of oral and parenteral dosage forms. Thus, the inhibitors of the present invention can be administered by injection, that is, intravenously, intramuscularly, intracutaneously, subcutaneously, intraduodenally, or intraperitoneally. Also, the compounds of the present invention can be administered by inhalation, for example, intranasally. Additionally, the inhibitors of the present invention can be administered transdermally. The following dosage forms may comprise as the active component an inhibitor of the present invention, or a pharmaceutically acceptable salt thereof.

For preparing pharmaceutical compositions from the inhibitors of the present invention, pharmaceutically acceptable carriers can be either solid or liquid. Solid form preparations include powders, tablets, pills, capsules, cachets, suppositories, and dispersible granules. A solid carrier can be one or more substances, which may also act as diluents, flavoring agents, binders, preservatives, tablet disintegrating agents, or an encapsulating material.

In powders, the carrier is a finely divided solid, which is in a mixture with the finely divided active component.

In tablets, the active component is mixed with the carrier having the necessary binding properties in suitable proportions and compacted in the shape and size desired.

The powders and tablets preferably contain from five or ten to about seventy percent of the active compound. Suitable carriers are magnesium carbonate, magnesium stearate, talc, sugar, lactose, pectin, dextrin, starch, gelatin, tragacanth, methylcellulose, sodium carboxymethylcellulose, a low melting wax, cocoa butter, and the like. The term "preparation" is intended to include the formulation of the active component with encapsulating material as a carrier providing a capsule in which the active component with or without other carriers, is surrounded by a carrier, which is thus in association with it. Similarly, cachets and lozenges are included. Tablets, powders, capsules, pills, cachets, and lozenges can be used as solid dosage forms suitable for oral administration.

For preparing suppositories, a low melting wax, such as a mixture of fatty acid glycerides or cocoa butter, is first melted, and the active component is dispersed homogeneously therein, as by stirring. The molten homogenous mixture is then poured into convenient sized molds, allowed to cool, and thereby to solidify.

Liquid form preparations include solutions, suspensions, and emulsions, for example, water or water propylene glycol solutions. For parenteral injection, liquid preparations can be formulated in solution in aqueous polyethylene glycol solution.

Aqueous solutions suitable for oral use can be prepared by dissolving the active component in water and adding suitable colorants, flavors, stabilizing and thickening agents as desired.

Aqueous suspensions suitable for oral use can be made by dispersing the finely divided active component in water with viscous material, such as natural or, synthetic gums, resins, methylcellulose, sodium carboxymethylcellulose, and other well-known suspending agents.

Also included are solid form preparations, which are intended to be converted, shortly before use, to liquid form preparations for oral administration. Such liquid forms include solutions, suspensions, and emulsions. These preparations may contain, in addition to the active component, colorants, flavors, stabilizers, buffers, artificial and natural sweeteners, dispersants, thickeners, solubilizing agents, and the like.

The pharmaceutical preparation is preferably in unit dosage form. In such form the preparation is divided into unit doses containing appropriate quantities of the active component. The unit dosage form can be a packaged preparation, the package containing discrete quantities of preparation, such as packeted tablets, capsules, and powders in vials or ampoules. Also, the unit dosage form can be a capsule, tablet, cachet, or lozenge itself, or it can be the appropriate number of any of these in packaged form.

The quantity of active component in a unit dose preparation may be varied or adjusted from 0.1 mg to 100 mg, preferably 0.5 mg to 100 mg, according to the particular application and the potency of the active component. The composition can, if desired, also contain other compatible therapeutic agents.

In therapeutic use as antagonists or as agents for the treatment of diseases; the compounds utilized in the pharmaceutical method of this invention are administered at the initial dosage of about 0.01 mg to about 100 mg/kg daily. A daily dose range of about 0.01 mg to about 10 mg/kg is preferred. The dosages, however, may be varied depending upon the requirements of the patient, the severity of the condition being treated, and the compound being employed. Determination of the proper dosage for a particular situation is within the skill of the art. Generally, treatment is initiated with smaller dosages, which are less than the optimum dose of the compound. Thereafter, the dosage is increased by small increments until the optimum effect under the circumstances is reached. For convenience, the total daily dosage may be divided and administered in portions during the day, if desired.

Examples of pharmaceutical preparations of the inhibitors of the present invention is described below. Such preparations can be administered to a human from one to six times a day for treatment of disease caused by a synergistic effect of a CSF on chemokine-mediated inflammation, osteoporosis, an autoimmune disease, or atherosclerosis.

EXAMPLE 4

Tablet Formulation:

Ingredients	Amount (mg)
Inhibitor of the invention	25
Lactose	50
Cornstarch (for mix)	10
Cornstarch (paste)	10
Magnesium stearate (1%)	5
Total	100

The inhibitor of the present invention, lactose, and cornstarch (for mix) are blended to uniformity. The cornstarch (for paste) is suspended in 200 mL of water and heated with stirring to form a paste. The paste is used to granulate the mixed powders. The wet granules are passed through a No. 8 hand screen and dried at

-35-

80°C. The dry granules are lubricated with the 1% magnesium stearate and pressed into a tablet.

EXAMPLE 5

Coated Tablets:

- 5 The tablets of Example 17 are coated in a customary manner with a coating of sucrose, potato starch, talc, tragacanth, and colorant.

EXAMPLE 6

Injection Vials:

- 10 The pH of a solution of 500 g of an inhibitor of the present invention and 5 g of disodium hydrogen phosphate is adjusted to pH 6.5 in 3 L of double-distilled water using 2 M hydrochloric acid. The solution is sterile-filtered, and the filtrate is filled into injection vials, lyophilized under sterile conditions, and aseptically sealed. Each injection vial contains 25 mg of an inhibitor of the present invention.

15 EXAMPLE 7

Suppositories:

A mixture of 25 g of an inhibitor of the present invention, 100 g of soya lecithin, and 1400 g of cocoa butter is fused, poured into molds, and allowed to cool. Each suppository contains 25 mg of an inhibitor of the present invention.

20 EXAMPLE 8

Solution:

- 25 A solution is prepared from 1 g of the an inhibitor of the present invention, 9.38 g of $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 28.48 g of $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, and 0.1 g benzalkonium chloride in 940 mL of double-distilled water. The pH of the solution is adjusted to pH 6.8 using 2 M hydrochloric acid. The solution is diluted to 1.0 L with double-distilled water and sterilized by irradiation. A 25-mL volume of the solution contains 25 mg of an inhibitor of the present invention.

-36-

EXAMPLE 9

Ointment:

Five hundred milligrams of an inhibitor of the present invention is mixed with 99.5 g of petroleum jelly under aseptic conditions. A 5-g portion of the ointment contains 25 mg of an inhibitor of the present invention.

EXAMPLE 10

Capsules:

Two kilograms of an inhibitor of the present invention are filled into hard gelatin capsules in a customary manner such that each capsule contains 25 mg of an inhibitor of the present invention.

EXAMPLE 11

Ampoules:

A solution of 2.5 kg of an inhibitor of the present invention is dissolved in 60 L of double-distilled water. The solution is sterile-filtered, and the filtrate is filled into ampoules. The ampoules are lyophilized under sterile conditions and aseptically sealed. Each ampoule contains 25 mg of an inhibitor of the present invention.

The embodiments of the present invention described above are hereupon claimed.

CLAIMS

What is claimed is:

1. An inhibitor of a colony stimulating factor (CSF), which inhibits the synergistic effect of said CSF on chemokine-mediated inflammation, osteoporosis, an autoimmune disease, or atherosclerosis, comprising an agent which binds to a CSF, an agent which inhibits expression of a CSF, an antagonist of a colony stimulating factor receptor (CSFR), an antibody directed to a CSF or a CSFR, or an agent which inhibits activation of a CSFR, or a pharmaceutically acceptable salt thereof.
2. The inhibitor of Claim 1 wherein the CSF is a monocyte-colony stimulating factor (M-CSF).
3. The inhibitor of Claim 1 wherein the chemokine is a beta-chemokine.
4. The inhibitor of Claim 1 wherein the CSF is an M-CSF, the chemokine is monocyte chemotactic protein-1 (MCP-1), and the inhibitor is an antibody directed to an M-CSF or an antibody directed to a monocyte-colony stimulating factor receptor (M-CSFR).
5. The inhibitor of Claim 1 wherein the CSF is an M-CSF, the chemokine is MCP-1, and the inhibitor is an antagonist of an M-CSFR.
6. The inhibitor of Claim 1 wherein the CSF is a granulocyte-colony stimulating factor (G-CSF).
7. The inhibitor of Claim 1 wherein the chemokine is an alpha-chemokine.
8. The inhibitor of Claim 1 wherein the CSF is a G-CSF, the chemokine is IL-8, and the inhibitor is an antibody directed to a G-CSF or an antibody directed to a granulocyte-colony stimulating factor receptor (G-CSFR).

-38-

9. The inhibitor of Claim 1 wherein the CSF is a G-CSF, the chemokine is IL-8, and the inhibitor is an antagonist of a G-CSFR.
10. The inhibitor of Claim 1 wherein the CSF is a granulocyte macrophage-colony stimulating factor (GM-CSF).
- 5 11. A pharmaceutical composition, comprising an inhibitor of a CSF which inhibits the synergistic effect of said CSF on chemokine-mediated inflammation, osteoporosis, an autoimmune disease, or atherosclerosis, or a pharmaceutically acceptable salt thereof, and a pharmaceutically acceptable carrier, diluent, or excipient.
- 10 12. A method of treating inflammation, osteoporosis, an autoimmune disease, or atherosclerosis, comprising administering to a mammal, in need thereof, a therapeutically effective amount of an inhibitor of a CSF which inhibits the synergistic effect of said CSF on chemokine-mediated inflammation, osteoporosis, an autoimmune disease, or atherosclerosis, or a
15 pharmaceutically acceptable salt thereof.
13. The method according to Claim 12 wherein the disease being treated is atherosclerosis.
14. The method according to Claim 12 wherein the disease being treated is sepsis.
- 20 15. The method according to Claim 12 wherein the disease being treated is asthma.
16. The method according to Claim 12 wherein the disease being treated is an autoimmune disease.
- 25 17. The method according to Claim 12 wherein the disease being treated is osteoporosis.

-39-

18. The method according to Claim 12 wherein the disease being treated is rheumatoid arthritis.
19. The method according to Claim 12 wherein the disease being treated is osteoarthritis.
- 5 20. A method for screening for an inhibitor of an M-CSF which inhibits the synergistic effect of said CSF on chemokine-mediated inflammation, osteoporosis, an autoimmune disease, or atherosclerosis, comprising analyzing an (M-CSF)-stimulated monocyte population using a Fluorescent Activated Cell Sorter technique.
- 10 21. The method according to Claim 20 wherein the (M-CSF)-stimulated monocyte population is analyzed in whole blood after red blood cell lysis.
22. The method according to Claim 20 wherein the screening method is a high throughput screening method.
- 15 23. The method according to Claim 20 wherein the (M-CSF)-stimulated monocyte population has also been stimulated by MCP-1.
24. The method according to Claim 23 wherein the (M-CSF)-stimulated monocyte population which has also been stimulated by MCP-1, is analyzed in whole blood after red blood cell lysis.
- 20 25. A method for screening for an inhibitor of a G-CSF which inhibits the synergistic effect of said CSF on chemokine-mediated inflammation, osteoporosis, an autoimmune disease, or atherosclerosis, comprising measuring binding of an (¹²⁵I) G-CSF to a G-CSFR in a (G-CSF)-stimulated neutrophil population.
- 25 26. The method according to Claim 25 wherein the screening method is a high throughput screening method.

-40-

27. A method for screening for an inhibitor of a GM-CSF which inhibits the synergistic effect of said CSF on chemokine-mediated inflammation, osteoporosis, an autoimmune disease, or atherosclerosis, comprising measuring binding of an (I^{125}) GM-CSF to a GM-CSFR in a (GM-CSF)-stimulated neutrophil population or analyzing a (GM-CSF)-stimulated monocyte population using a Fluorescent Activated Cell Sorter technique.
28. A method for screening for an inhibitor of a CSF which inhibits the synergistic effect of said CSF on chemokine-mediated inflammation, osteoporosis, an autoimmune disease, or atherosclerosis, the method comprising:
- Step (a) Obtaining CSFR cDNA and corresponding (I^{125})-CSF;
- Step (b) Cloning the CSFR cDNA of Step (a) into a vector;
- Step (c) Stably transfecting the vector of Step (b) into a hematopoietic cell line that resembles circulating leukocytes;
- Step (d) Quantitating the transfected vector of Step (c) and measuring the binding of said (I^{125})-CSF; and
- Step (e) Screening agents for inhibition of CSF activity using a binding assay comprising the transfected vector of Step (c) and said (I^{125})-CSF.
29. A method for screening for an inhibitor of an M-CSF which inhibits the synergistic effect of said CSF on chemokine-mediated inflammation, osteoporosis, an autoimmune disease, or atherosclerosis, comprising measuring binding of an (I^{125}) M-CSF to an M-CSFR in an (M-CSF)-stimulated monocyte population.
30. The method according to Claim 29 wherein the M-CSFR is a soluble M-CSFR.

ABSTRACT

- 5 A hematopoietic factor called "colony stimulating factor" (CSF) is capable of synergizing the attracting capabilities of chemokines and of inducing the accumulation and/or activation in vitro and in vivo of key components of inflammatory responses. Various types of agents that inhibit or otherwise hinder the production, release or activity of CSF could be used therapeutically in the treatment of ischemia and other inflammatory diseases, such as autoimmune disease, and various chronic inflammatory diseases such as rheumatoid arthritis and psoriasis.

09885259 .022301

THIS PAGE BLANK (USPTO)

Fig. 1. G-CSF Synergizes IL-8 Induced Neutrophil Chemotaxis

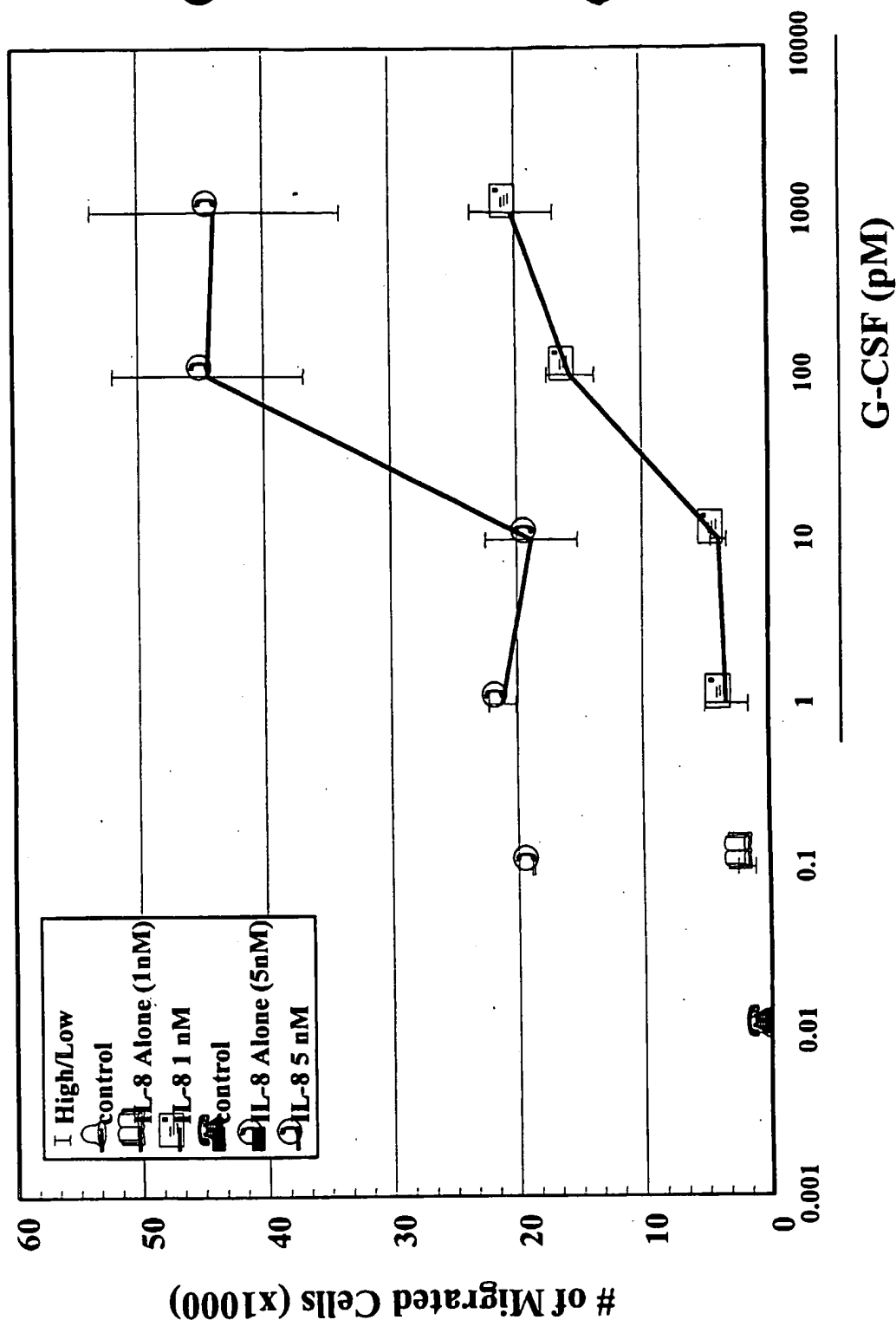


Figure 2
GM-CSF Synergizes IL8 Induced PMN Chemotaxis

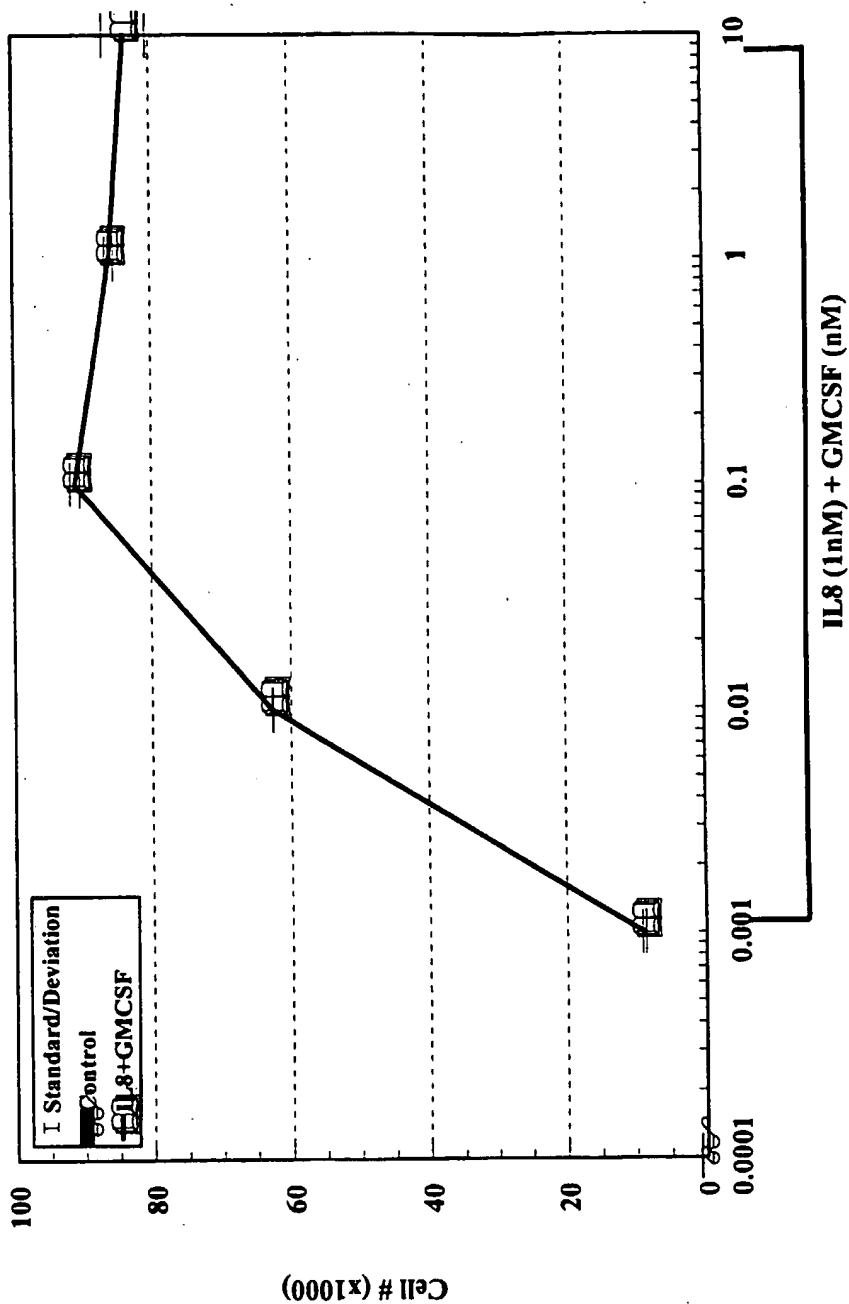
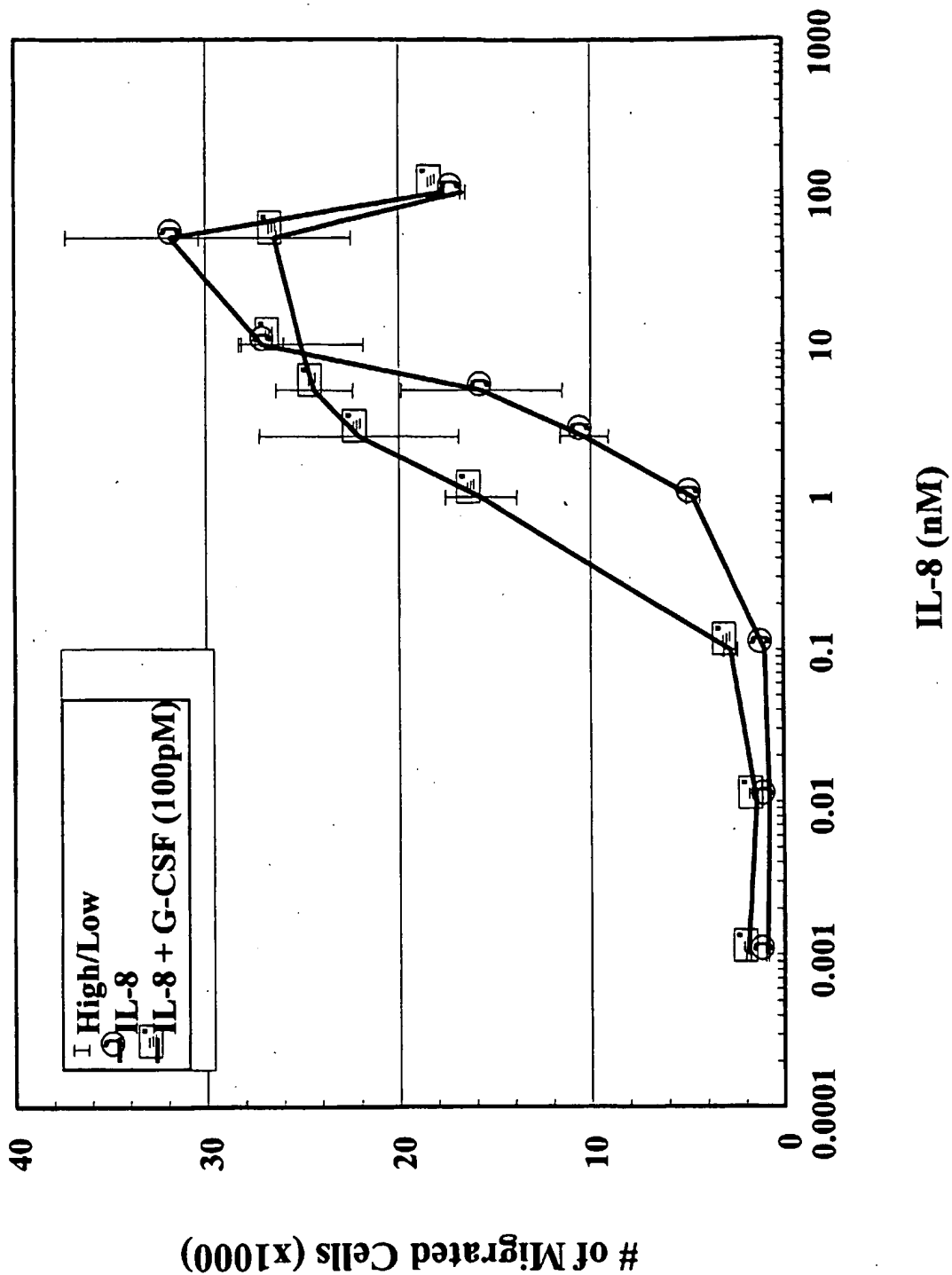


Fig. 3. Dose Response Curve for IL-8 with Constant G-CSF (100 pM)



**Fig. 4. GCSF Does not Synergize f-MLP
Induced Neutrophil Chemotaxis**

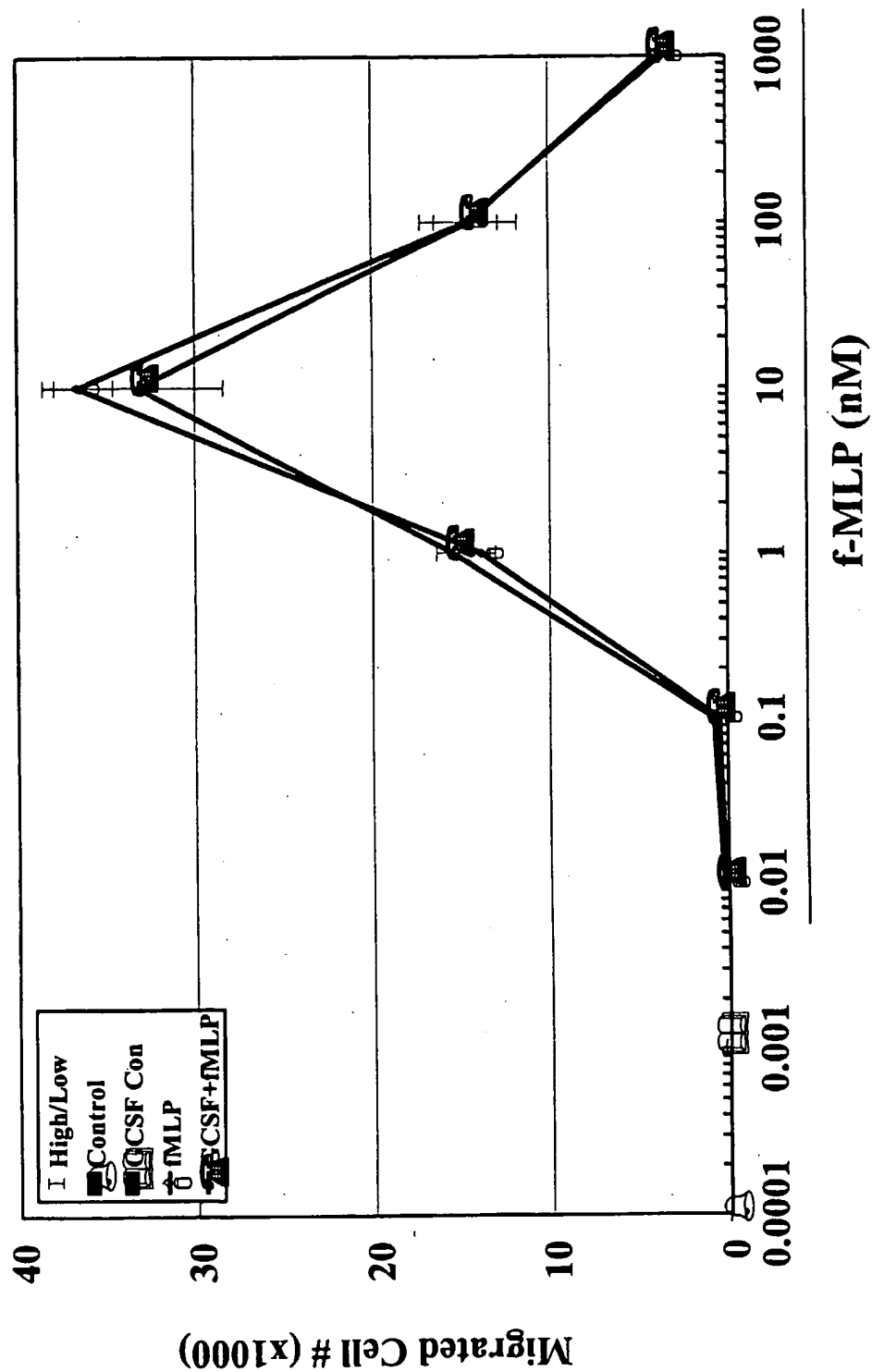


Fig.5. G-CSF enhances *in vivo* neutrophil intradermal recruitment

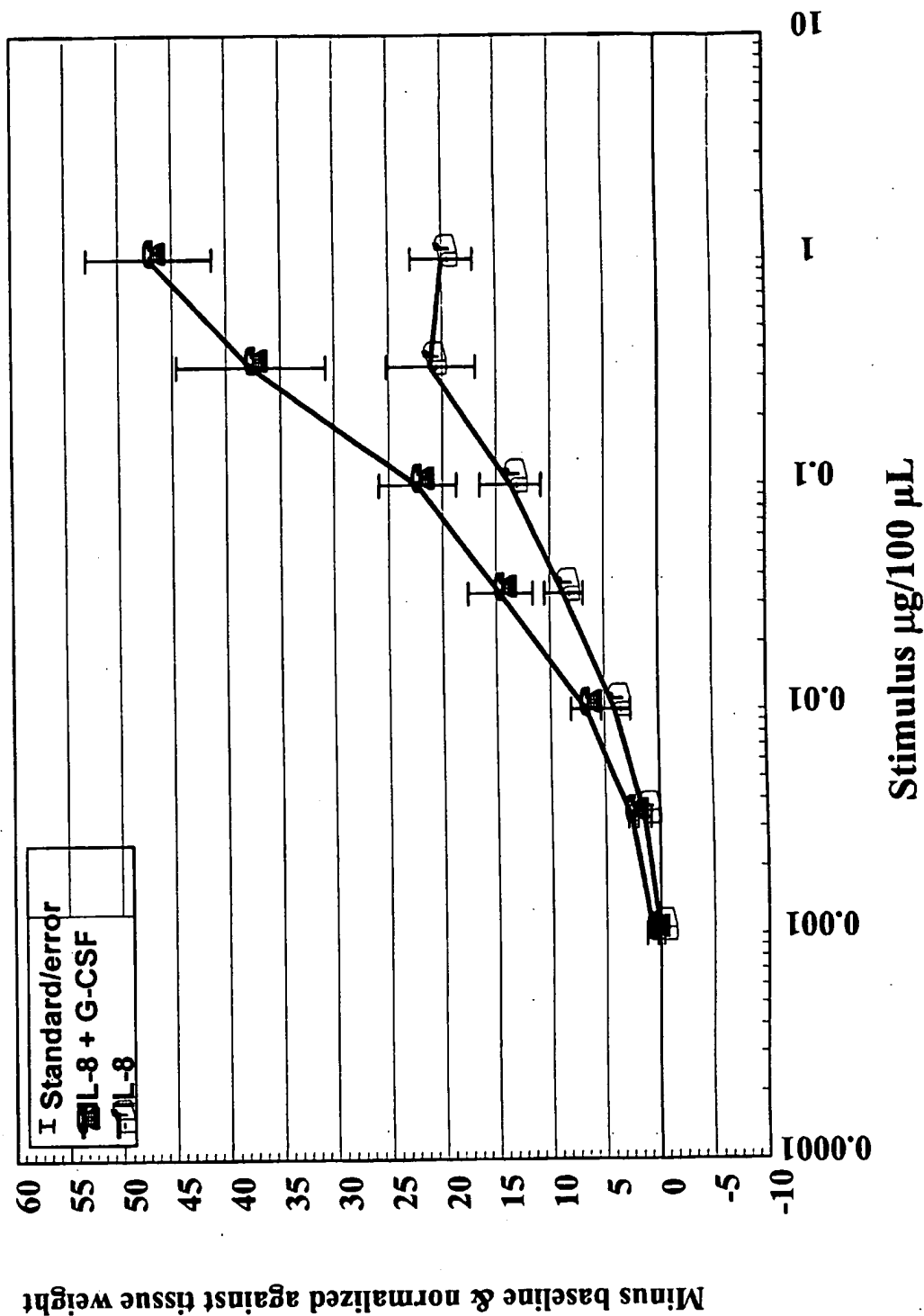


Fig. 6. Binding of ^{125}I G-CSF on PMN

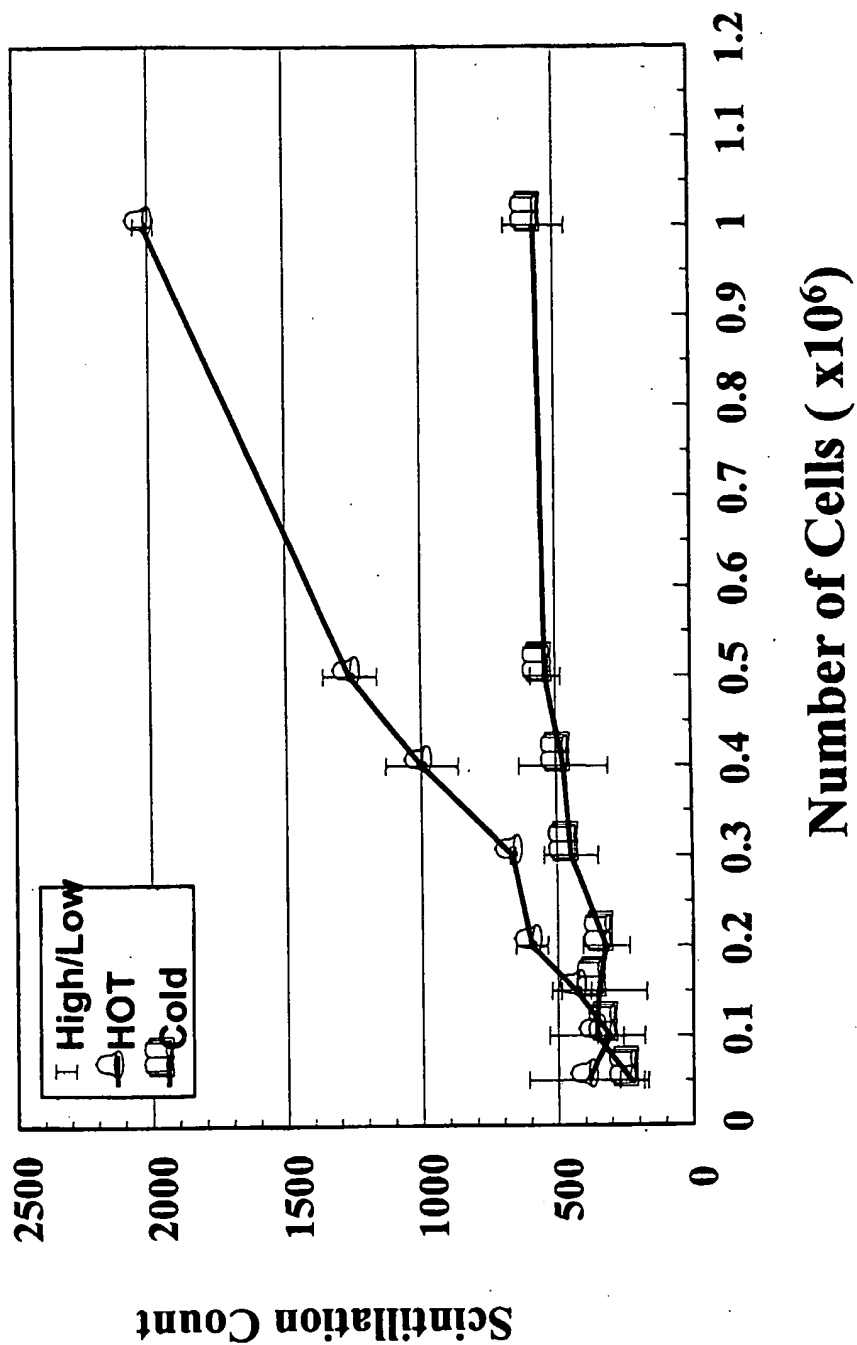


Fig. 7. G-CSF Neutralizing Antibody Inhibits G-CSF Synergized Chemotaxis

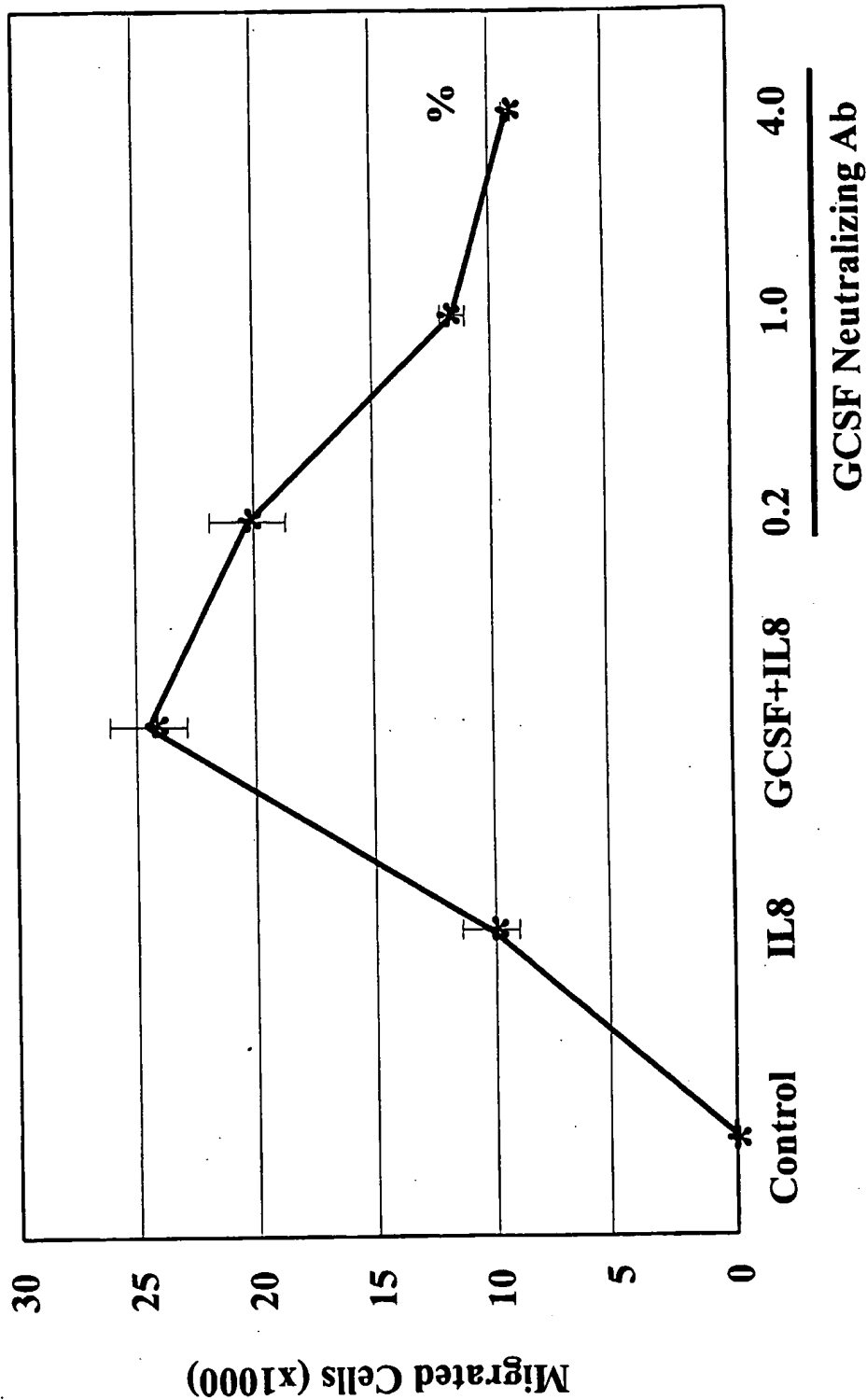
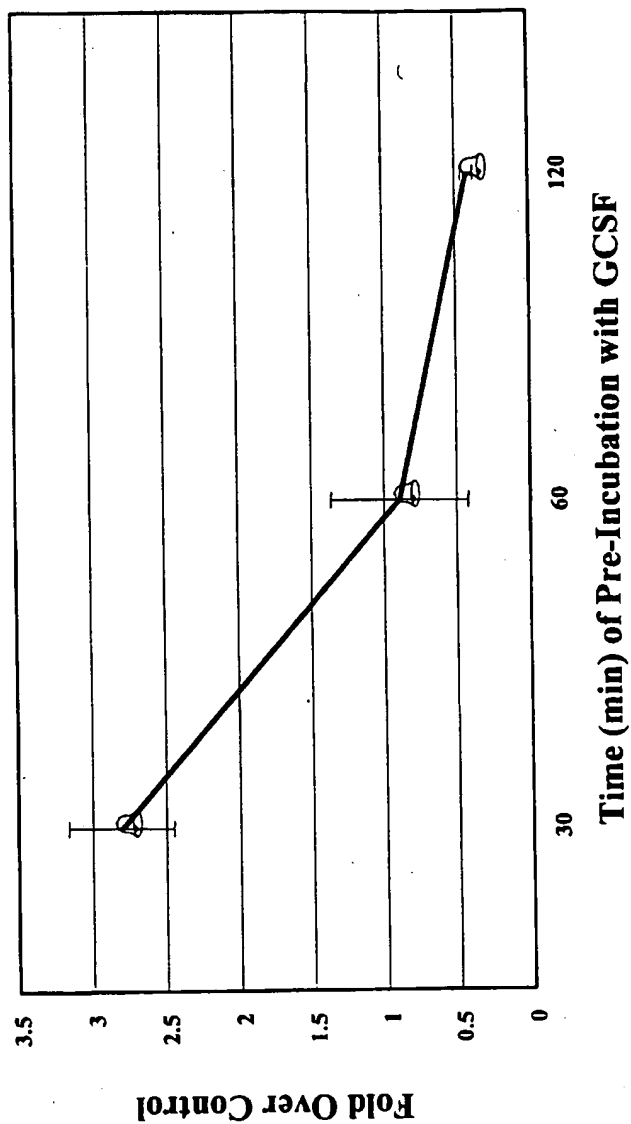
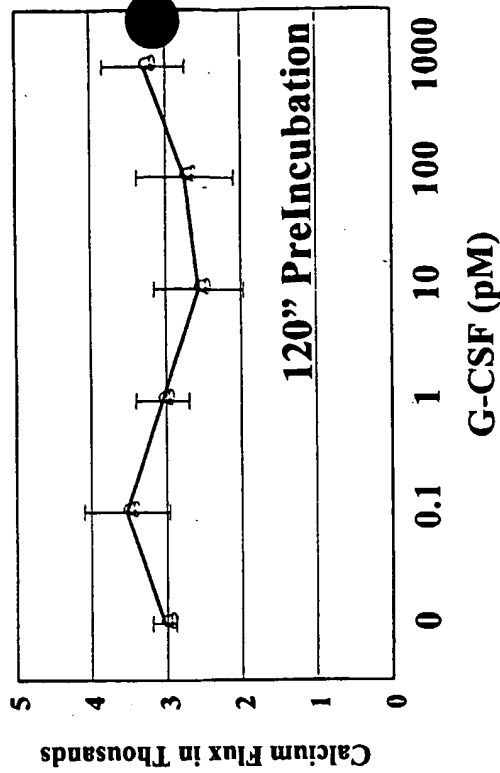
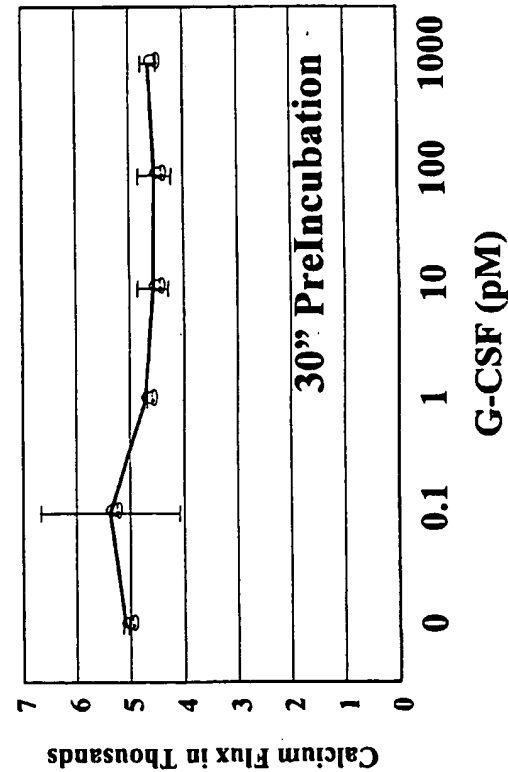
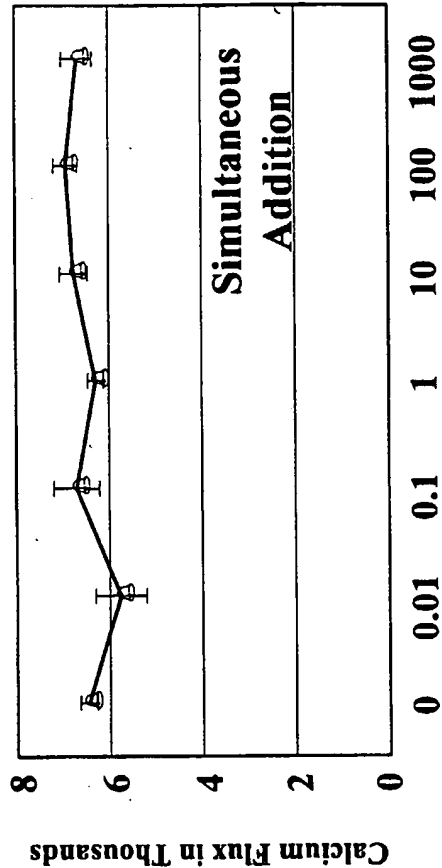


Fig. 8. G-CSF Pre-Incubation Decreases Neutrophil Response to IL-8



Cells were preincubated with G-CSF for respective time periods and subsequently treated with 1nM of IL-8

Fig. 9. G-CSF Does not Alter IL-8 Induced Calcium Flux



I High/Low
GCSF + IL8
N.S. - 0 pM GCSF
N.S. - 1000 pM GCSF

GCSF (pM, 3 h incubation)	Scintillation Count (x Thousands)
0	~8.5
1	~8.8
10	~9.2
100	~8.5
1000	~6.5

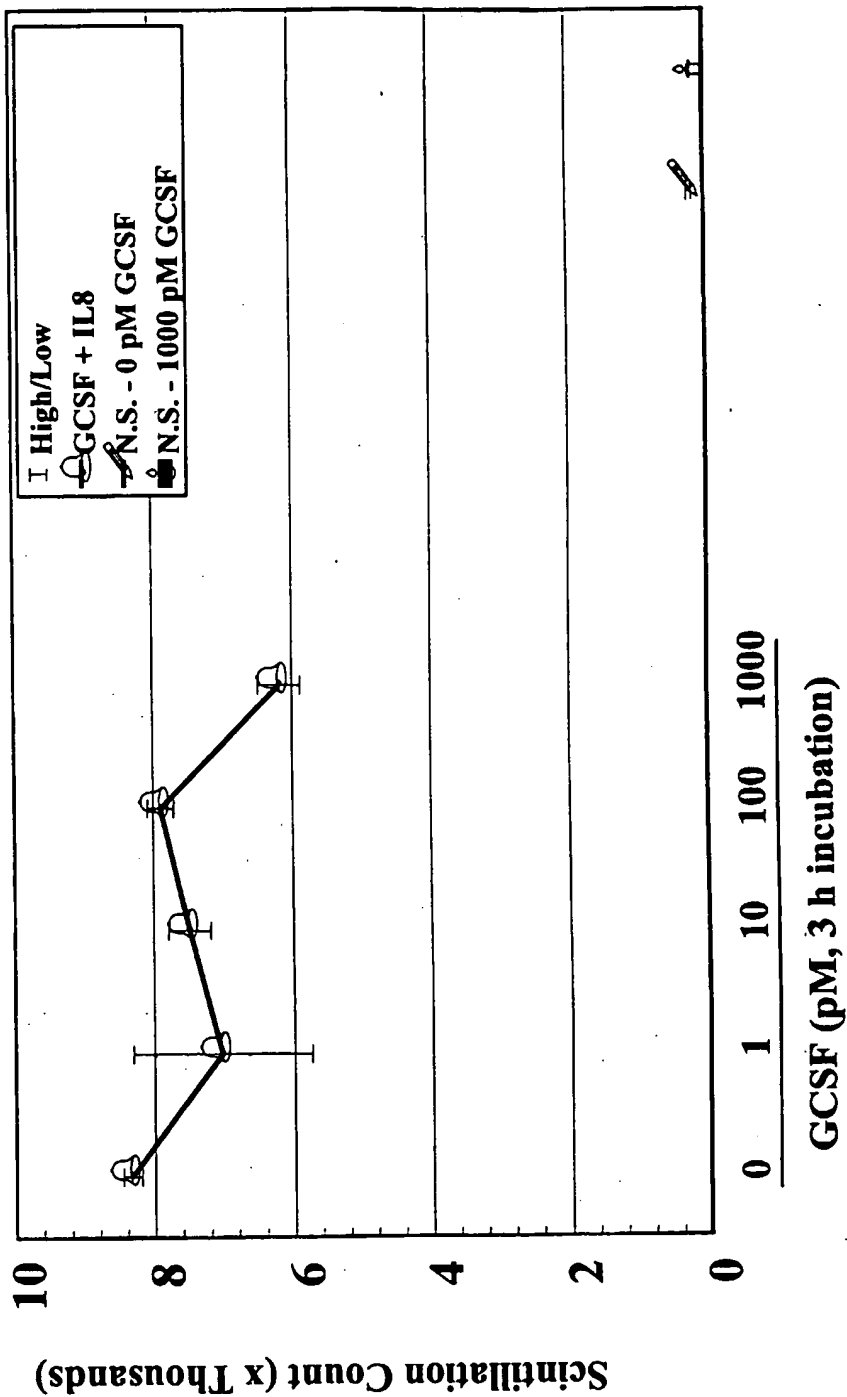
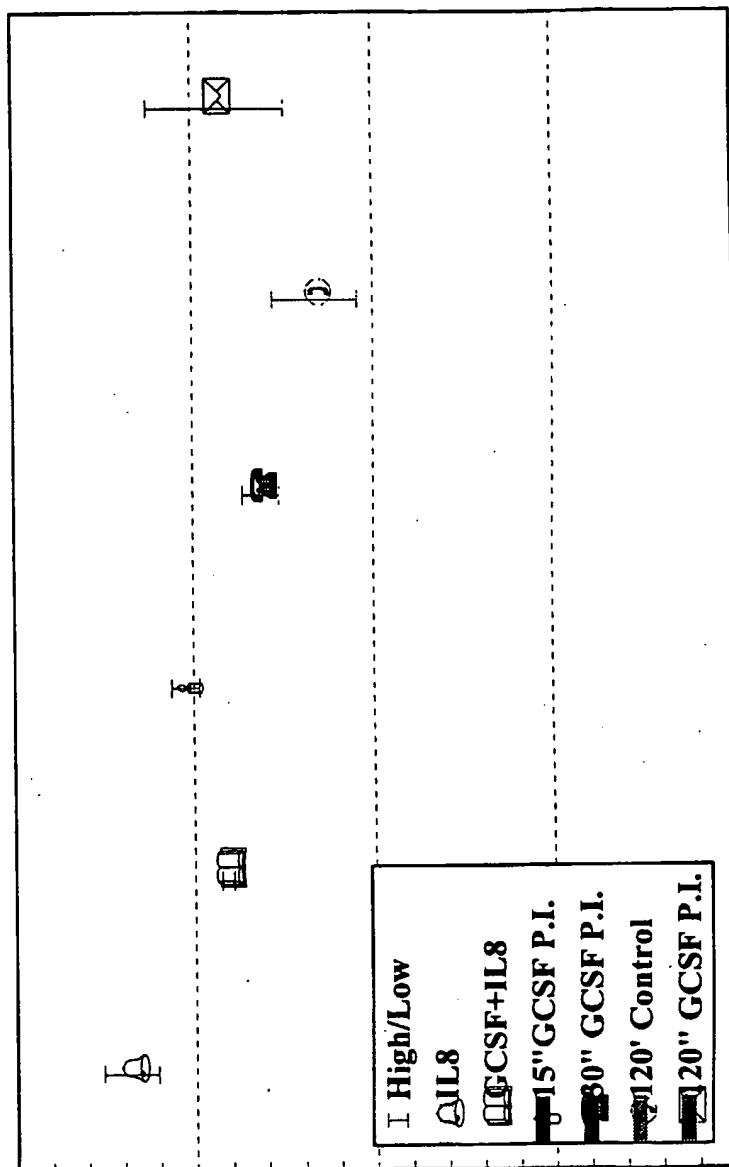


Fig. 11. G-CSF Preincubation Does not Alter IL-8 Binding on Neutrophils



100 pM of G-CSF was incubated simultaneously or pretreated for the respective time periods

Fig. 12 G-CSF Pre-Incubation Alters PMN Response to LI-8

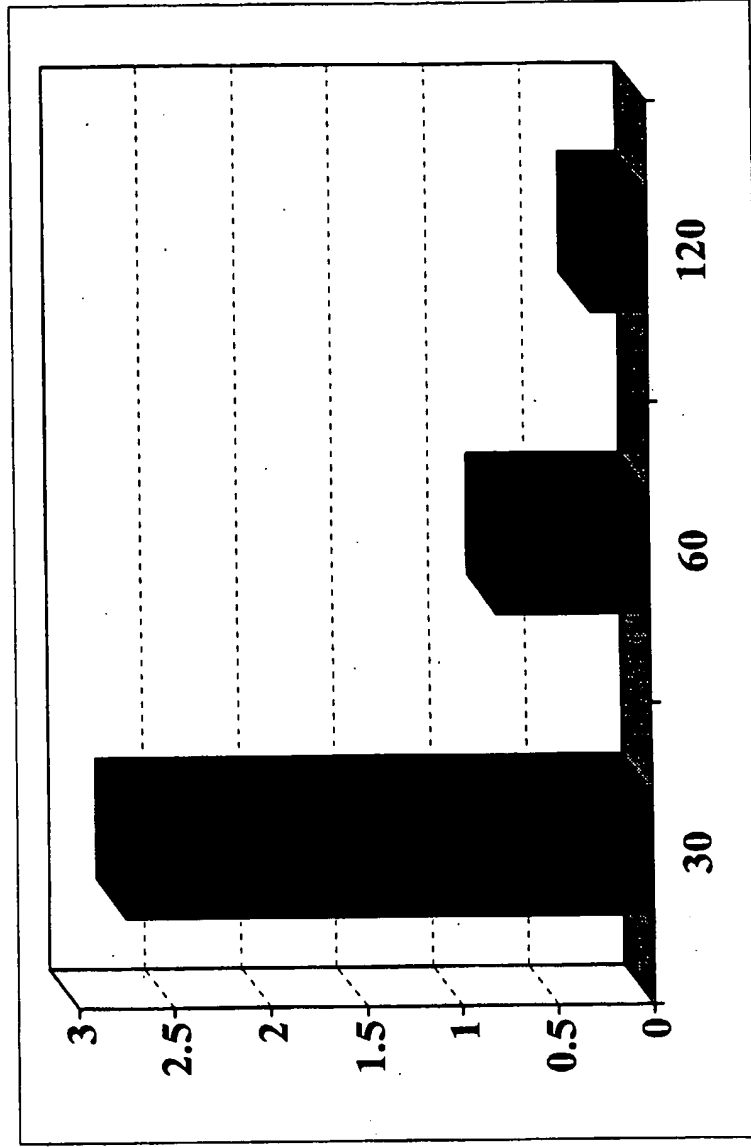
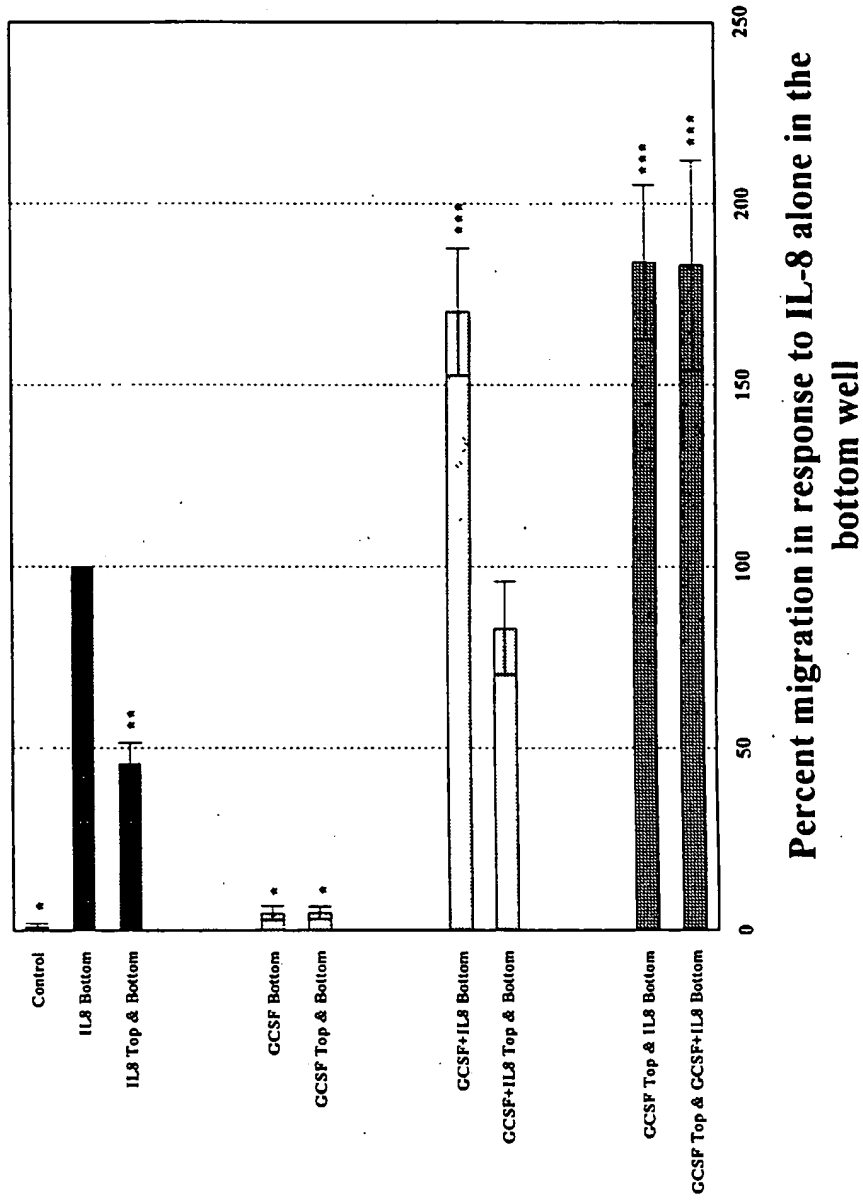


Figure 13: G-CSF potentiates both chemokinetic and chemotactic effects of IL-8



பெரியகடல், பெருங்கடல்

2

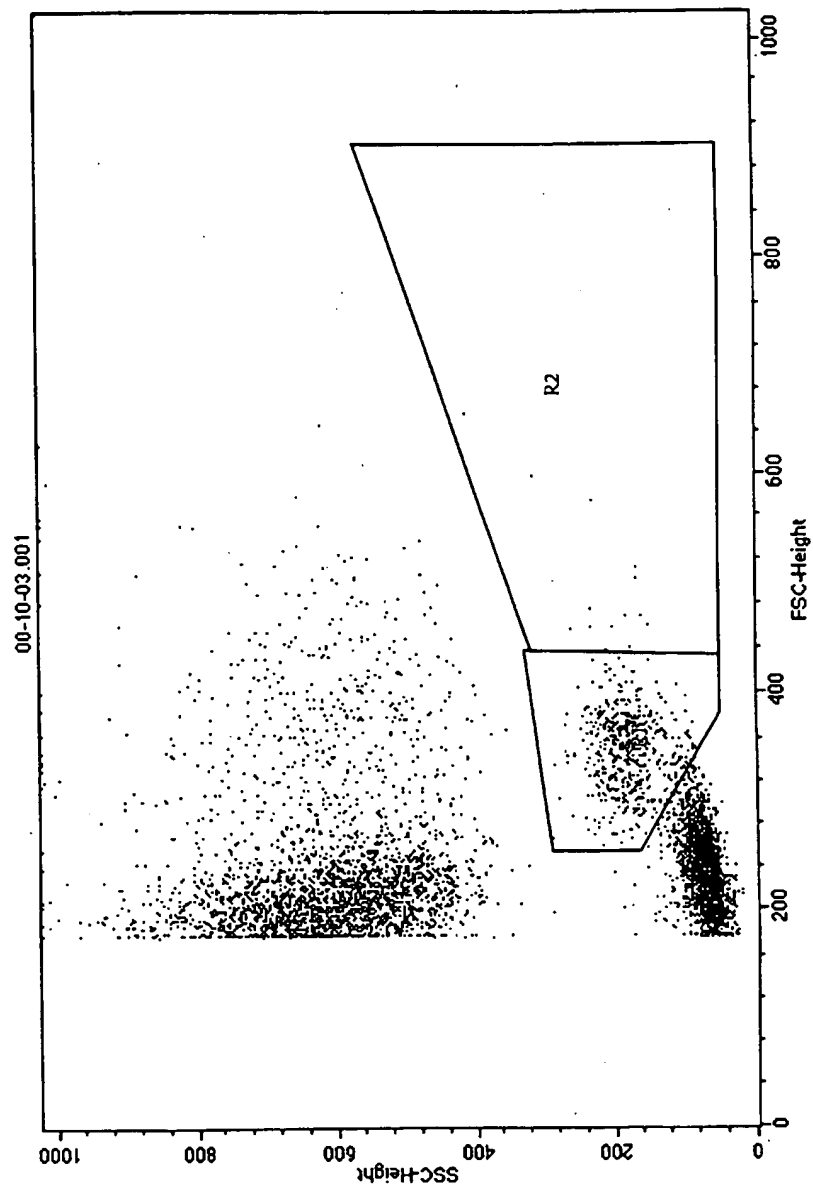


Figure 15: FACS Dot plot of FSC vs. SSC from unstimulated and MCP-1 stimulated human whole blood

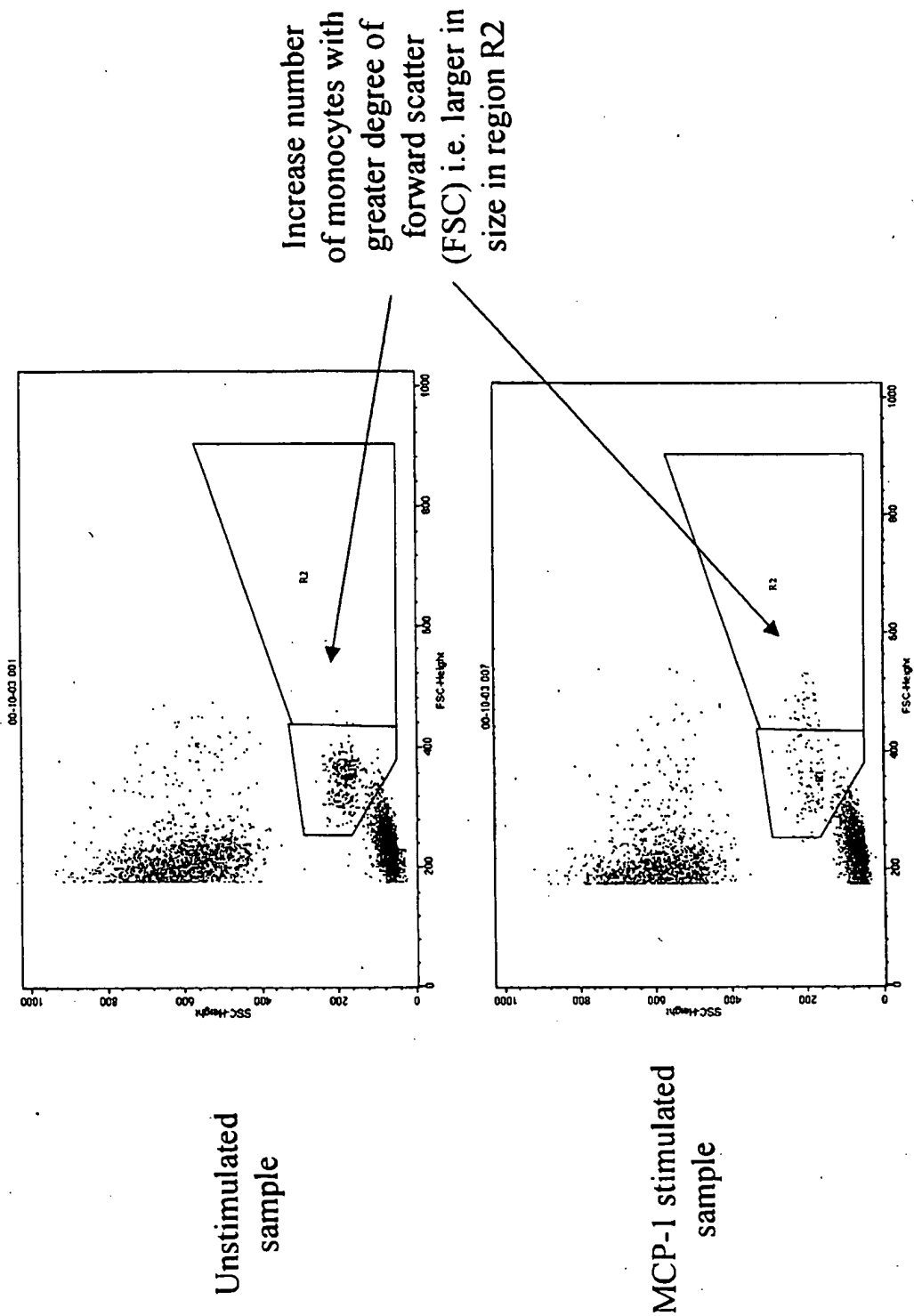


Figure 16: Time course of FSC changes in response to MCP-1 stimulation

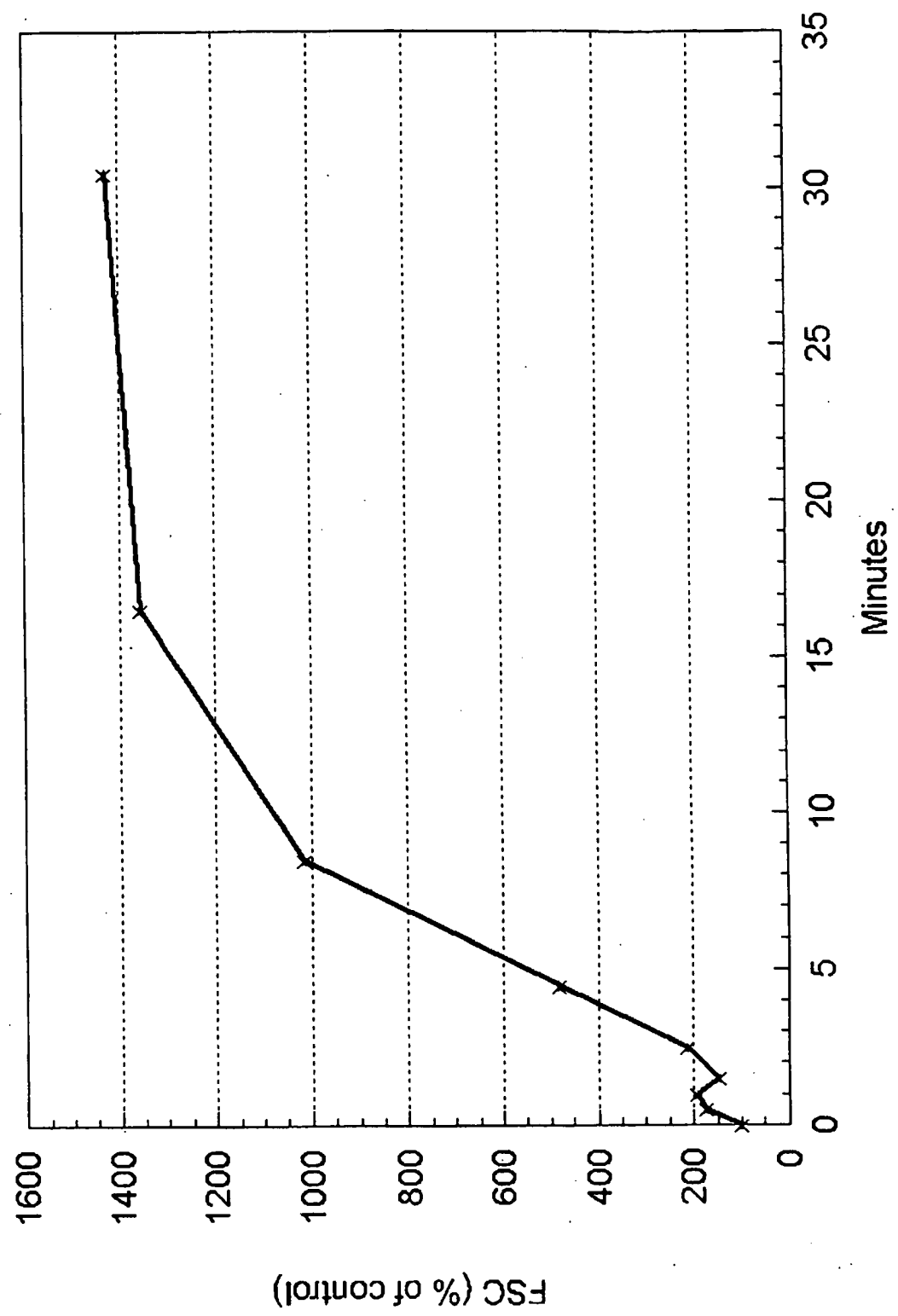


Figure 17: Dose-response curve to MCP-1 stimulation

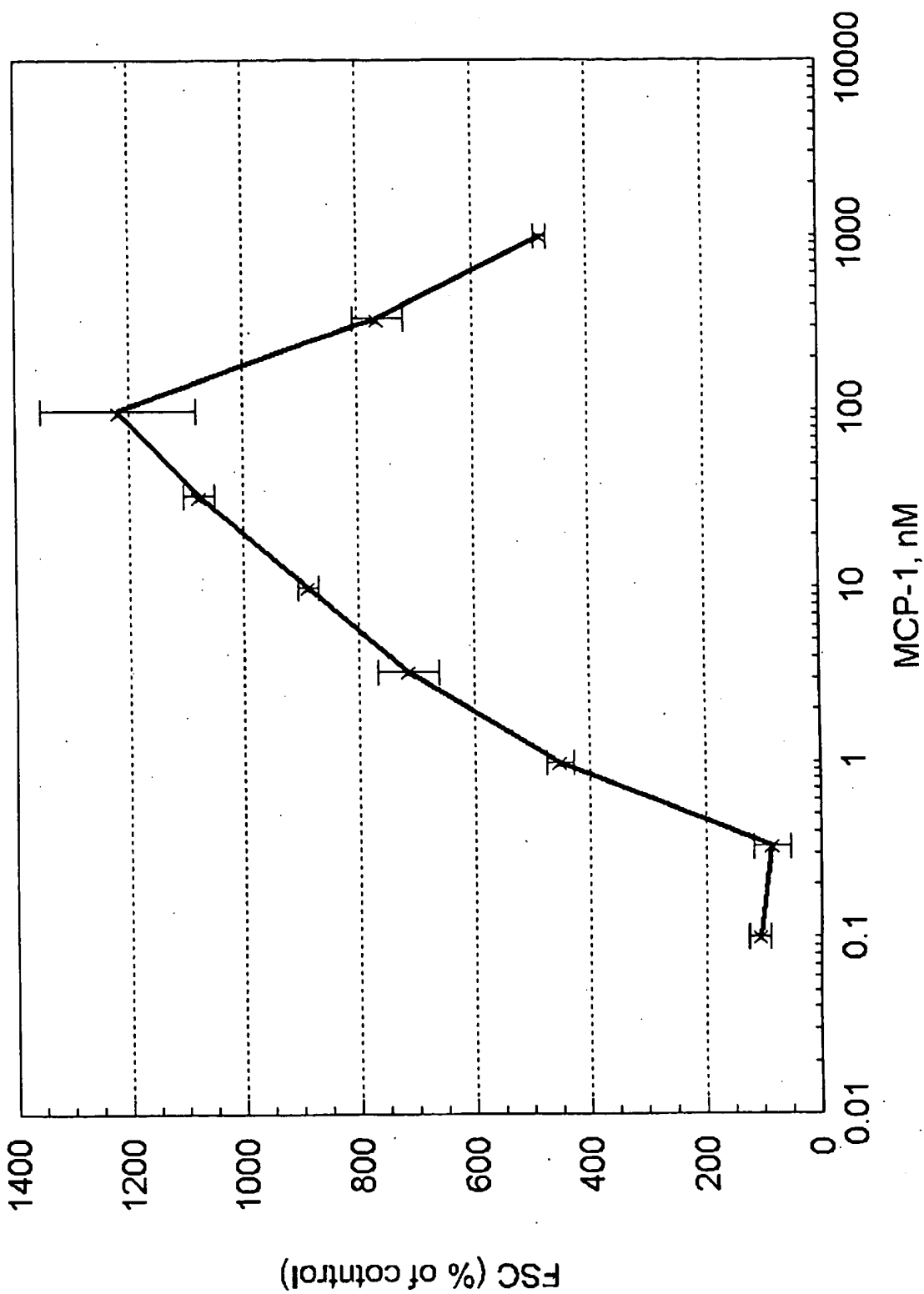


Figure 18: Inhibitory effects of anti-CCR2 antibody on MCP-1 stimulated FSC changes

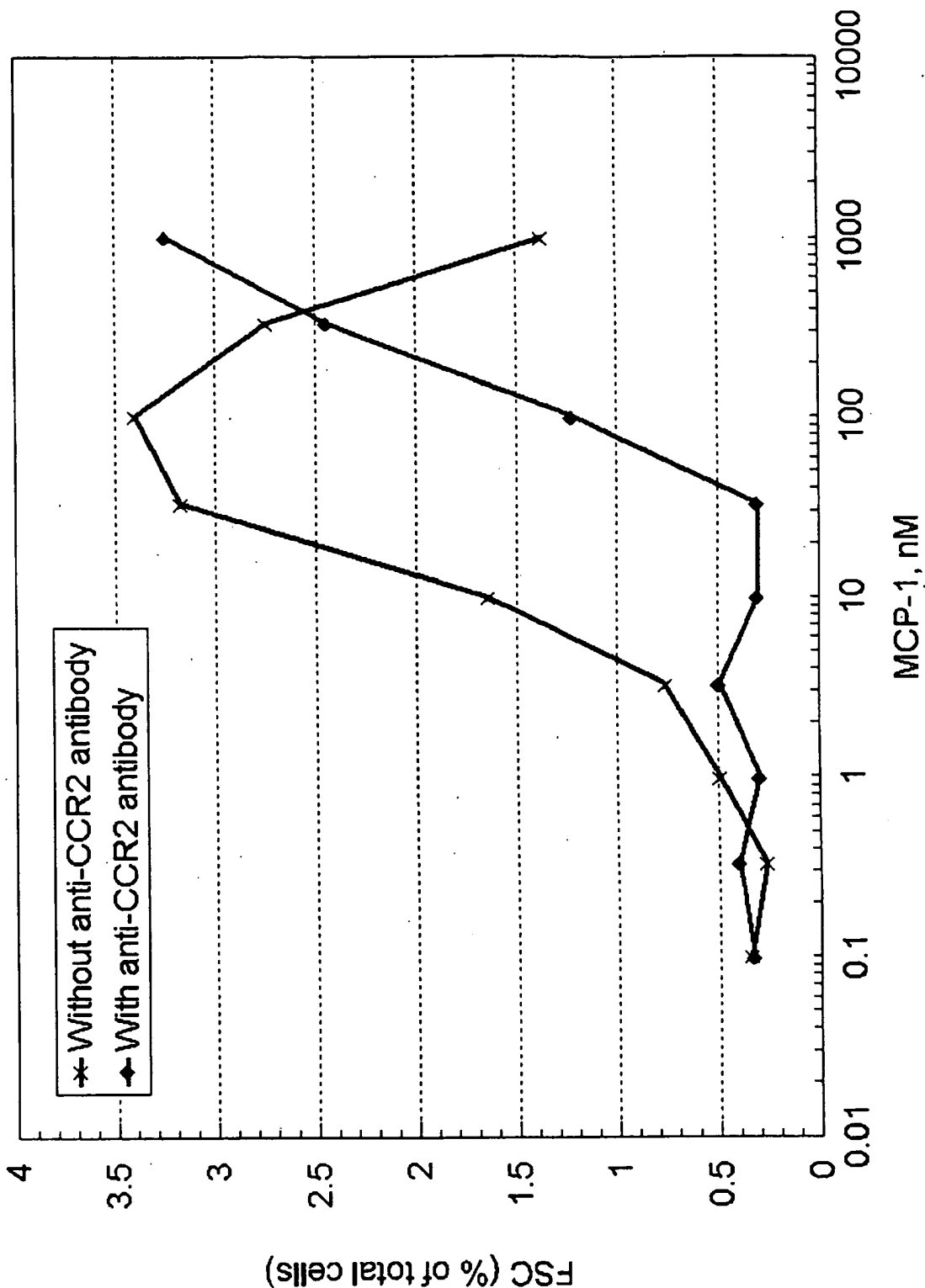
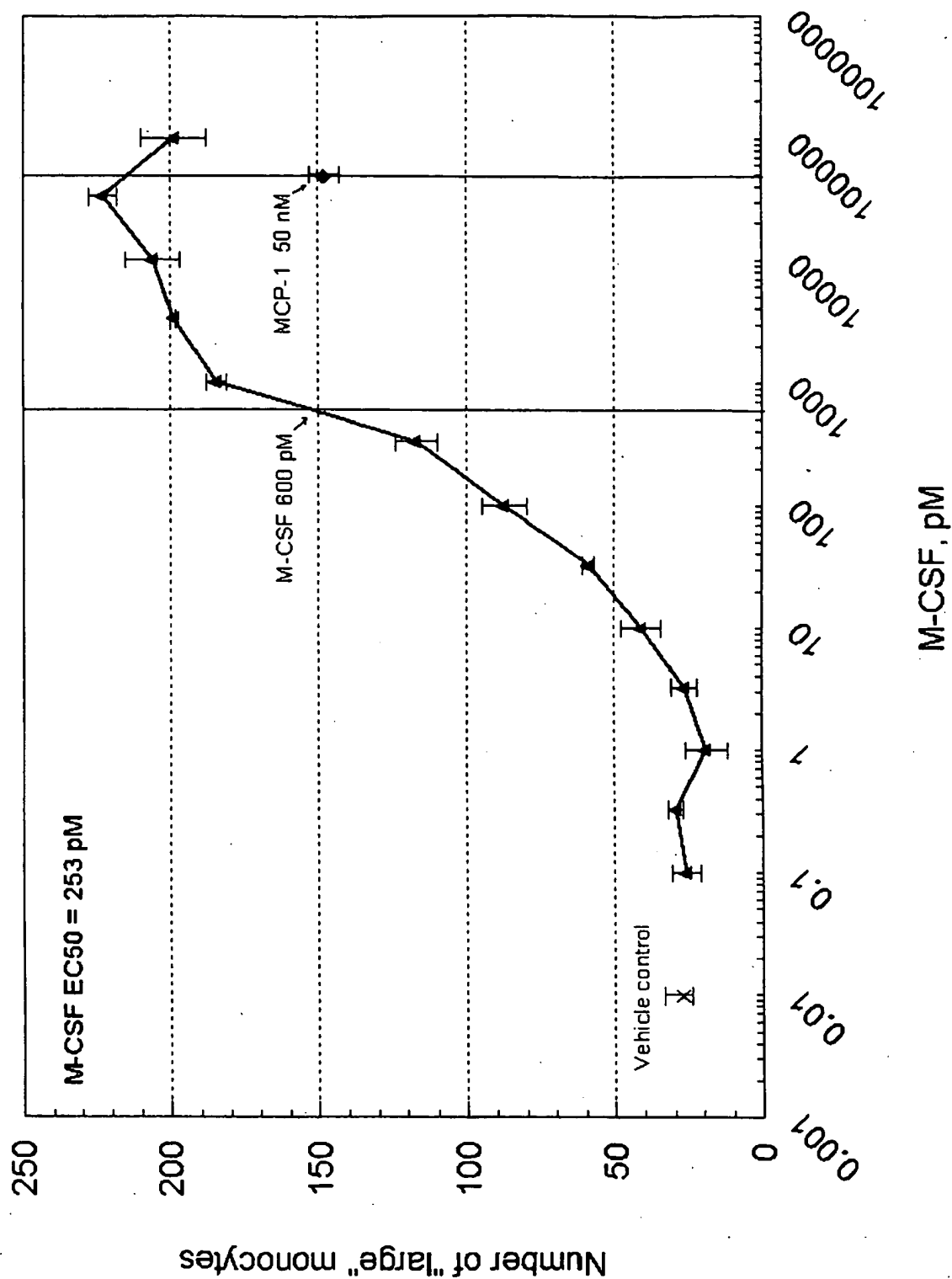


Figure 19: Effect of M-CSF on human monocyte shape change - comparison to MCP-1



202203160209

Figure 20: M-CSF specificity - effect on human neutrophil shape change

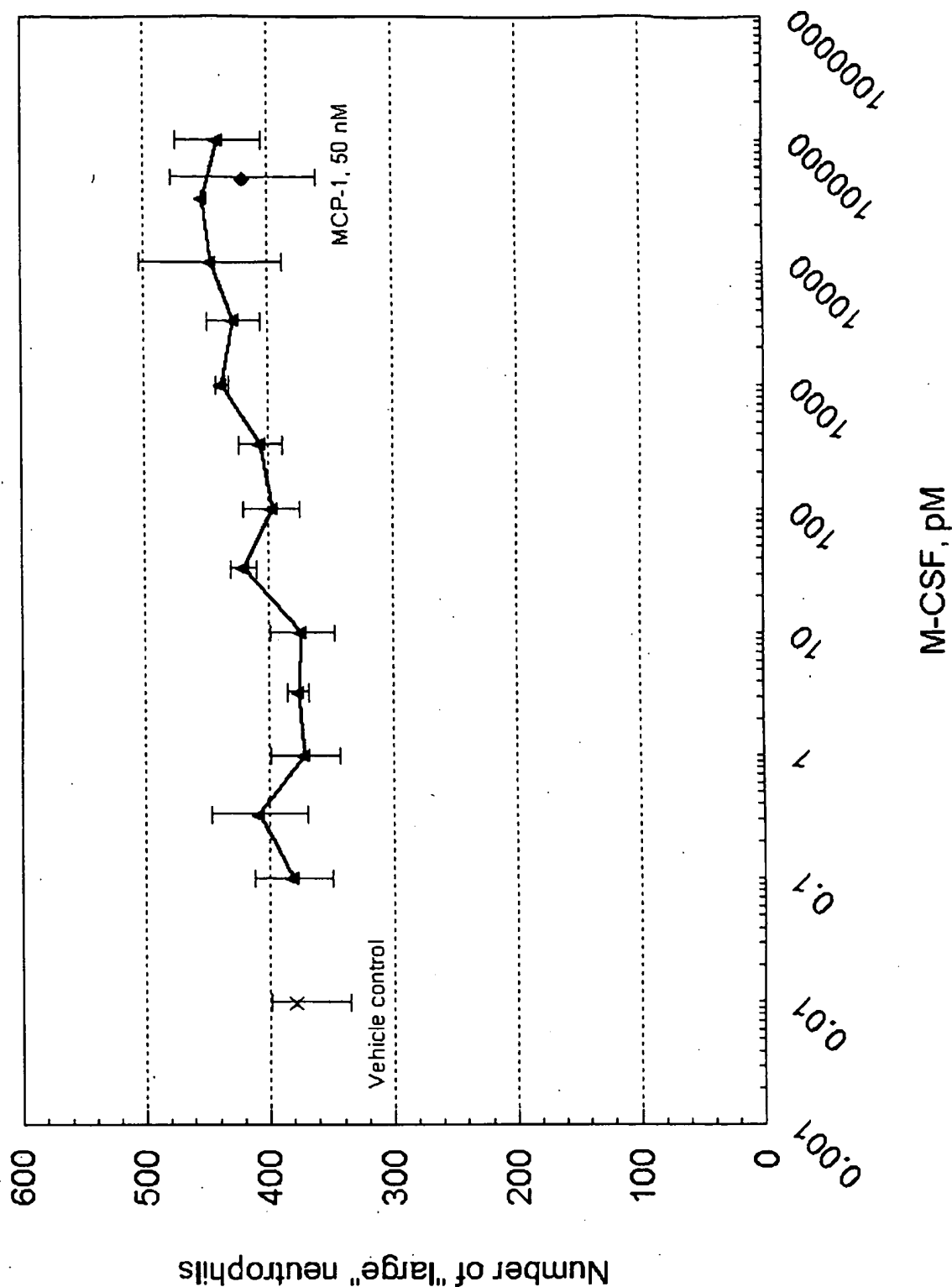
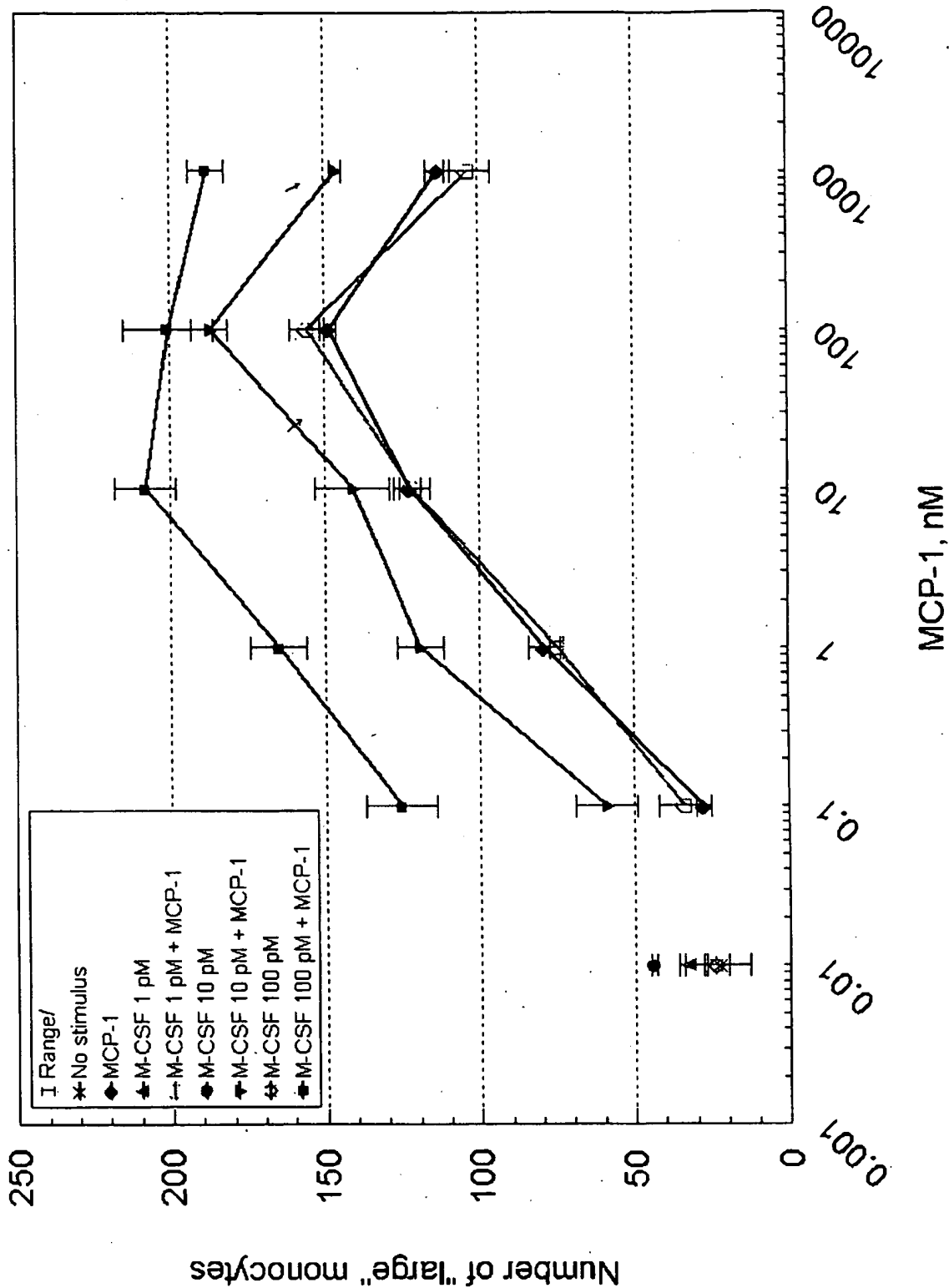


Figure 1 is a line graph showing the number of "large" monocytes (Y-axis, 0 to 250) versus MCP-1 concentration (X-axis, log scale from 0.007 to 10000 nM). The graph includes data for various conditions: No stimulus, MCP-1, M-CSF 1 pM, M-CSF 10 pM, M-CSF 100 pM, and M-CSF 100 pM + MCP-1. The number of "large" monocytes generally increases with MCP-1 concentration, with the highest values observed at 100 pM M-CSF + MCP-1.

MCP-1, nM	No stimulus	MCP-1	M-CSF 1 pM	M-CSF 10 pM	M-CSF 100 pM	M-CSF 100 pM + MCP-1
0.007	~20	~20	~20	~20	~20	~20
0.01	~20	~20	~20	~20	~20	~20
0.03	~20	~20	~20	~20	~20	~20
0.1	~20	~20	~20	~20	~20	~20
0.3	~20	~20	~20	~20	~20	~20
1	~20	~20	~20	~20	~20	~20
3	~20	~20	~20	~20	~20	~20
10	~20	~20	~20	~20	~20	~20
30	~20	~20	~20	~20	~20	~20
100	~20	~20	~20	~20	~20	~20
300	~20	~20	~20	~20	~20	~20
1000	~20	~20	~20	~20	~20	~20
3000	~20	~20	~20	~20	~20	~20
10000	~20	~20	~20	~20	~20	~20



Search results as of: 05-30-2006::09:57:11 E.T.

Bibliographic Data

Application Number:	60/190,842	Customer Number:	-
Filing or 371 (c) Date:	03-20-2000	Status:	Provisional Application Expired
Application Type:	Provisional	Status Date:	09-22-2001
Examiner Name:	-	Location:	FILE REPOSITORY (FRANCONIA)
Group Art Unit:	-	Location Date:	05-30-2001
Confirmation Number:	6617	Earliest Publication No:	-
Attorney Docket Number:	A0000326L1-01MJA	Earliest Publication Date:	-
Class / Subclass:	-	Patent Number:	-
First Named Inventor:	Madhav Devalaraja , Ann Arbor, MI	Issue Date of Patent:	-

Title of Invention:	Colony stimulating factors as synergizers of chemokine induced cell
---------------------	---

[Close Window](#)



Search results as of: 05-30-2006::09:57:30 E.T.

Transaction History	
Date	Transaction Description
09-22-2001	Set Application Status
05-06-2000	Application Dispatched from OIPE
05-06-2000	Correspondence Address Change
03-30-2000	IFW Scan & PACR Auto Security Review
03-20-2000	Initial Exam Team nn

Close Window

Search results as of: 05-30-2006::09:57:51 E.T.

This application is officially maintained in paper form. The document images below do NOT comprise the complete contents of the application file wrapper. To View: Click the desired Document Description. To Download and Print: Check the desired document(s) and click StartDownload.

Mail Room Date 	Document Description 	Page Count
03-20-2000	Miscellaneous Incoming Letter	38

[Close Window](#)

Search results as of: 05-30-2006::09:58:19 E.T.

Parent Continuity Data

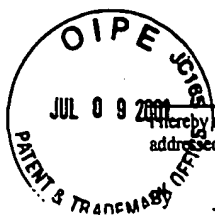
Description	Parent Number	Parent Filing or 371(c) Date	Parent Status	Patent Number
-------------	------------------	---------------------------------	------------------	------------------

**No Parent Continuity Data
Found.**

Child Continuity Data

09/885,259 filed on 02-23-2001 which is Pending claims the benefit of 60/190,842

[Close Window](#)



I hereby certify that this correspondence is being deposited with the United States Postal Service as Express mail in an envelope addressed to: Hon. Commissioner of Patents and Trademarks, Washington, D.C. 20231 on this 60 day of July 2001.

Seth H. Jacobs
(Signature of person mailing)

Seth H. Jacobs
(Typed or printed name of person)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICATION OF: Madhav N. Devalaraja and Joseph
E. Low

APPLICATION NO: 60/270948

: Examiner: Not yet assigned

FILING DATE: February 23, 2001

TITLE: INHIBITORS OF COLONY STIMULATING
FACTORS

Hon. Commissioner of Patents and Trademarks
Washington, D.C. 20231

Sir:

PETITION FOR CONVERSION OF PROVISIONAL APPLICATION
TO NON-PROVISIONAL APPLICATION UNDER 37 C.F.R. §1.53(c)(3)

Applicant(s) respectfully request that the present provisional application be converted to a non-provisional application pursuant to 37 C.F.R. §1.53(3)(c).

Priority of earlier filed provisional application serial no. 60/190,842, filed March 20, 2000 is claimed under 35 U.S.C. §119(e). A preliminary amendment to the present specification, adding a claim to such priority, is included herein.



720
gp
#26

I hereby certify that this correspondence is being deposited with the United States Postal Service as Express mail in an envelope addressed to:
Hon. Commissioner of Patents and Trademarks, Washington, D.C. 20231 on this 68 day of July 2001.

By

Seth H. Jacobs
(Signature of person mailing)
Seth H. Jacobs

(Typed or printed name of person)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN RE APPLICATION OF: Madhav N. Devalaraja and :
Joseph E. Low

APPLICATION SERIAL NO.: Non-provisional : Examiner: Not yet assigned
application converted from serial no. 60/270,948

FILING DATE: February 23, 2001 : Group Art Unit:

TITLE: INHIBITORS OF COLONY :
STIMULATING FACTORS

Box Amendment
Commissioner of Patents and Trademarks
Washington, D.C. 20231

Sir:

PRELIMINARY AMENDMENT

Sirs:

Kindly amend the above referenced application as follows:

IN THE SPECIFICATION:

At page 1, line 1 of the specification, insert:

"This application claims priority of Serial No. 60/190,842, filed March 20, 2000."

Adjustment date: 08/08/2002 GDUCKETT
03/11/2002 JTIPPETT 00000001 161445 60270948
01 FC:101 710.00 CR

IN THE CLAIMS:

Add the following claims:

31-32. A method of treating inflammation in a mammal comprising administering to
said mammal an effective amount of an m-CSF inhibitor

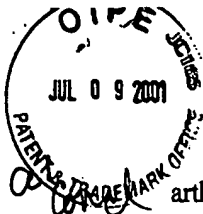
32-33. The method of claim 32 wherein said inhibitor is an antibody.

USERS\DOCS\LA21952\LP\SHJ\N\5011.DOC / 169012 / PC18174A PRELIMINARY AMENDMENT

Adjustment date: 08/08/2002 GDUCKETT
09/05/2001 JTIPPETT 00000001 161445 60270948
02 FC:102 480.00 CR
03 FC:103 234.00 CR

03/11/2002 JTIPPETT 00000001 161445 60270948
01 FC:101 710.00 CR

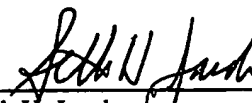
09/05/2001 JTIPPETT 00000001 161445 60270948
01 FC:102 130.00 CH
02 FC:102 480.00 CH
03 FC:103 234.00 CH



33
81
The method of claim 32 wherein said inflammation is associated with
arthritis.--

Respectfully submitted,

Date: _____



Seth H. Jacobs
Attorney for Applicant(s)
Reg. No. 32,140

Pfizer, Inc
Patent Department, 20th Floor
235 East 42nd Street
New York, NY 10017-5755
(212) 733-3678



UNDER SECRETARY OF COMMERCE FOR INTELLECTUAL PROPERTY AND
DIRECTOR OF THE UNITED STATES PATENT AND TRADEMARK OFFICE
WASHINGTON, D.C. 20231
www.uspto.gov

September 5, 2001

Paper # 3

Claude F. Purchase, Jr.
Warner-Lambert Company
2800 Plymouth Road
Ann Arbor MI 48105

In re Application of:	Devalaraja, et al.	:	DECISION GRANTING
Application No.:	60/270,948	:	PETITION
Filed:	February 23, 2001	:	
Attorney Docket No.:	A000026L2-01CFP	:	

This is a decision on your petition under 37 CFR 1.53(b)(1), received in the Patent and Trademark Office on July 09, 2001, to convert the above identified application to a non-provisional application under 35 U.S.C. 111 (a) and 37 CFR 1.53(b)(1).

The petition is granted.

The application will be processed in the Office of Initial Patent Examination (OIPE) as a non-provisional application under 35 U.S.C. 111(a) and 37 CFR 1.53(b)(1), including the assignment of a new non-provisional application number.

The non-provisional application serial number is 09/885,259. The filing receipt for the non-provisional application will be mailed in due course.

Janice Tippet, Program Assistant
Office of Initial Patent Examination
(703) 308-0910



COPY OF PAPERS
ORIGINALLY FILED

Receipt

PC18174A

I hereby certify that this correspondence is being deposited with the United States Postal Service as first-class mail in an envelope addressed to: Commissioner of Patents and Trademarks, Washington, D.C. 20231 on this 22 day of April, 2002.

By

Seth H. Jacobs
(Signature of person mailing)

Seth H. Jacobs

(Typed or printed name of person)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICATION OF: Madhav N. Devalaraja and

Joseph E. Low

APPLICATION NO.: 09/885,259

Examiner: Not Yet assigned

FILING DATE: February 23, 2001

TITLE: INHIBITORS OF COLONY
STIMULATING FACTORS

Commissioner of Patents
Washington, D.C. 20231

Sir:

RECEIVED

AUG 22 2002

TECH CENTER 1600/2900

REQUEST FOR CORRECTED FILING RECEIPT

Applicant requests that the filing receipt for the above-identified application be corrected in claiming priority of:

Provisional 60/190,842, filed 3/20/00

Respectfully submitted,

Date: _____

Seth H. Jacobs
Seth H. Jacobs
Attorney for Applicant(s)
Reg. No. 32,140

Pfizer, Inc
Patent Department, 20th Floor
150 East 42nd Street
New York, NY 10017-5612
(212) 733-3678



PC18174A

I hereby certify that this correspondence is being deposited with the United States Postal Service as first-class mail in an envelope addressed to: Commissioner of Patents and Trademarks, Washington, D.C. 20231 on this 6th day of May, 2002.

By


(Signature of person mailing)
Seth H. Jacobs

(Typed or printed name of person)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**APPLICATION OF: Madhav N. Devalaraja and****Joseph E. Low****APPLICATION NO.: 09/885,259****Examiner: Not Yet Assigned****FILING DATE: February 23, 2001****TITLE: INHIBITORS OF COLONY
STIMULATING FACTORS****BOX MISSING PARTS****Commissioner of Patents
Washington, D.C. 20231**

Sir:


REQUEST FOR CORRECTED FILING RECEIPT

Applicant requests that the filing receipt for the above-identified application be corrected in claiming priority of:

Provisional 60/190,842, filed 3/20/00

Respectfully submitted,

Date: _____




Seth H. Jacobs
Attorney for Applicant(s)
Reg. No. 32,140

Pfizer, Inc
Patent Department, 20th Floor
150 East 42nd Street
New York, NY 10017-5612
(212) 733-3678

**COPY OF PAPERS
ORIGINALLY FILED**

Search results as of: 05-30-2006::20:29:17 E.T.

This application is officially maintained in electronic form. To View: Click the desired Document Description. To Download and Print: Check the desired document(s) and click StartDownload.

Mail Room Date 	Document Description 	Page Count
05-15-2006	Notice of Allowance and Fees Due (PTOL-85)	3
05-15-2006	Notice of Allowance and Fees Due (PTOL-85)	3
05-15-2006	Examiner Interview Summary Record (PTOL - 413)	1
05-15-2006	List of References cited by applicant and considered by examiner	1
05-15-2006	Issue Information including classification, examiner, name, claim, renumbering, etc.	1
05-15-2006	Search information including classification, databases and other search related notes	1
03-21-2006	Fee Worksheet (PTO-875)	2
03-21-2006	Amendment - After Non-Final Rejection	1
03-21-2006	Claims	2
03-21-2006	Applicant Arguments/Remarks Made in an Amendment	8
03-21-2006	Transmittal to TC	3
03-21-2006	Extension of Time	1
03-21-2006	Information Disclosure Statement (IDS) Filed	1
03-21-2006	NPL Documents	4
09-21-2005	Non-Final Rejection	8
09-21-2005	List of references cited by examiner	1
09-21-2005	NPL Documents	3
09-21-2005	Search information including classification, databases and other search related notes	1
09-21-2005	Index of Claims	1
06-29-2005	Fee Worksheet (PTO-875)	1
06-29-2005	Claims Worksheet (PTO-2022)	1
06-29-2005	Amendment Submitted/Entered with Filing of CPA/RCE	1
06-29-2005	Claims	2
06-29-2005	Applicant Arguments/Remarks Made in an Amendment	3
06-29-2005	Request for Continued Examination (RCE)	2
04-08-2005	Final Rejection	6
04-08-2005	List of References cited by applicant and	2

	considered by examiner	
04-08-2005	Search information including classification, databases and other search related notes	1
04-08-2005	Index of Claims	1
02-22-2005	Amendment - After Non-Final Rejection	1
02-22-2005	Claims	1
02-22-2005	Applicant Arguments/Remarks Made in an Amendment	4
02-22-2005	Miscellaneous Incoming Letter	2
02-22-2005	Information Disclosure Statement (IDS) Filed	3
02-22-2005	Foreign Reference	34
02-22-2005	Foreign Reference	135
02-22-2005	NPL Documents	2
02-22-2005	NPL Documents	1
02-22-2005	NPL Documents	2
12-21-2004	Change of Address	1
11-22-2004	Non-Final Rejection	6
11-22-2004	List of references cited by examiner	1
11-22-2004	Foreign Reference	51
11-22-2004	List of References cited by applicant and considered by examiner	1
11-22-2004	Index of Claims	1
11-22-2004	Search information including classification, databases and other search related notes	1
09-15-2004	Fee Worksheet (PTO-875)	1
09-15-2004	Amendment - After Non-Final Rejection	2
09-15-2004	Claims	1
09-15-2004	Applicant Arguments/Remarks Made in an Amendment	3
09-15-2004	Extension of Time	2
09-15-2004	Rule 131 or 132 Affidavits	18
09-15-2004	Information Disclosure Statement (IDS) Filed	1
09-15-2004	NPL Documents	12
09-15-2004	NPL Documents	7
09-15-2004	Miscellaneous Incoming Letter	1
08-16-2004	Examiner Interview Summary Record (PTOL - 413)	3
04-07-2004	Non-Final Rejection	6

04-07-2004	List of References cited by applicant and considered by examiner	1
04-07-2004	List of references cited by examiner	1
04-07-2004	NPL Documents	7
04-07-2004	Search information including classification, databases and other search related notes	1
04-07-2004	Index of Claims	1
01-28-2004	Amendment After Final	4
01-28-2004	Claims	3
01-28-2004	Applicant Arguments/Remarks Made in an Amendment	3
01-28-2004	Information Disclosure Statement (IDS) Filed	1
01-28-2004	NPL Documents	17
01-28-2004	Extension of Time	1
07-28-2003	Final Rejection	6
07-28-2003	List of references cited by examiner	1
07-28-2003	List of References cited by applicant and considered by examiner	1
06-16-2003	Amendment - After Non-Final Rejection	4
06-16-2003	Specification	1
06-16-2003	Claims	3
06-16-2003	Applicant Arguments/Remarks Made in an Amendment	9
06-16-2003	Drawings	22
06-16-2003	Extension of Time	1
06-16-2003	NPL Documents	2
06-16-2003	NPL Documents	5
05-07-2003	Power of Attorney (may include Associate POA)	2
01-28-2003	Non-Final Rejection	10
01-28-2003	Notice of Formal Drawings Required	2
01-28-2003	List of references cited by examiner	1
01-28-2003	List of References cited by applicant and considered by examiner	1
12-09-2002	Examiner's search strategy and results	6
11-15-2002	Amendment - After Non-Final Rejection	1
11-15-2002	Claims	1
11-15-2002	Applicant Arguments/Remarks Made in an Amendment	2

11-15-2002	Information Disclosure Statement (IDS) Filed	1
11-15-2002	NPL Documents	8
11-15-2002	NPL Documents	10
11-15-2002	NPL Documents	7
11-15-2002	NPL Documents	3
11-15-2002	NPL Documents	10
11-15-2002	NPL Documents	9
11-15-2002	NPL Documents	8
09-10-2002	Final Rejection	7
05-15-2002	Miscellaneous Incoming Letter	3
05-15-2002	Oath or Declaration filed	3
04-30-2002	Miscellaneous Incoming Letter	1
04-30-2002	Change of Address	1
03-11-2002	Miscellaneous Action with SSP	1
10-01-2001	Change of Address	1
09-05-2001	Petition Decision	1
07-09-2001	Amendment - After Non-Final Rejection	1
07-09-2001	Claims	1
07-09-2001	Petition Entered	1
02-23-2001	Issue Information including classification, examiner, name, claim, renumbering, etc.	1
02-23-2001	Search information including classification, databases and other search related notes	1
02-23-2001	Index of Claims	1
02-23-2001	Transmittal letter	2
02-23-2001	Drawings	21
02-23-2001	Specification	37
02-23-2001	Claims	4
02-23-2001	Abstract	1
02-23-2001	Fee Worksheet (PTO-875)	2
02-23-2001	Fee Worksheet (PTO-875)	1
02-23-2001	Transmittal letter	2
02-23-2001	Specification	37
02-23-2001	Claims	4
02-23-2001	Abstract	1
02-23-2001	Drawings	21

[Close Window](#)

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

☒ **BLACK BORDERS**

☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**

☐ **FADED TEXT OR DRAWING**

☐ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**

☐ **SKEWED/SLANTED IMAGES**

☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**

☐ **GRAY SCALE DOCUMENTS**

☐ **LINES OR MARKS ON ORIGINAL DOCUMENT**

☐ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**

☐ **OTHER:** _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.